

# **Fermentative Production of Value-Added Products from Sorghum Bran**

**Amina M. Ahmed El-Imam**

**B.Sc., M.Sc.**

Thesis submitted to the University of Nottingham  
for the degree of Doctor of Philosophy

May, 2017.

## Abstract

Studies were undertaken to investigate the potential for using sorghum bran from a traditional wet-milling process as a raw material for the fermentative production of value-added products, namely ethanol and itaconic acid. This was deemed of importance because renewable ethanol sources are required to replace environmentally unfriendly fossil-based fuels, while renewable itaconic acid could replace fossil-based industrial monomers if an economically viable source can be found. Emphasis was placed on optimising both yields (g/l) and substrate conversion, as a function of the theoretical maximum possible, for both compounds.

The compositions of sorghum white bran (WB) and red bran (RB) were first elucidated to determine their suitability for downstream fermentation uses. Results revealed that the brans appeared to contain adequate components for biomass growth, with approximate carbohydrate, protein, lipid and ash contents of 69 %, 16 %, 3 %, 2 % and 70 %, 17 %, 4 %, 1 % found in WB and RB, respectively. Second, methods to obtain glucose-rich hydrolysates (for later use in fermentations) from the sorghum bran were investigated. It was found that amylolytic enzymes could produce enzyme hydrolysates (WBEH and RBEH) with glucose contents ranging from 48 g/l to 61 g/l. Alternatively, the brans could be digested to simple sugars using 3 %  $\text{H}_2\text{SO}_4$  which converted up to 70.5 % of the bran into glucose, with dilute acid hydrolysates (WBDAH and RBDAH) having glucose contents ranging from 48 g/l - 57 g/l. Levels of the common fermentation inhibitors hydroxymethylfurfural, furaldehyde and vanillin were relatively low in all sorghum bran hydrolysates. Spot plate tests and phenotypic microarrays revealed that several yeast species metabolised and thrived on the hydrolysates. Ethanol mini-fermentations were successful with *Kluyveromyces marxianus* attaining the highest 88.9 % of theoretical maximum ethanol on RBEH, with other yeast strains also producing high yields. Production of itaconic acid by fermentation with 46 isolates of *Aspergillus terreus* was then attempted. Screening experiments on a defined glucose medium revealed that IA production levels were not normally distributed in nature. The highest producers were employed in 25 ml fermentation using the sorghum hydrolysates as a feedstock but the yields were found to be low compared to use of the defined glucose medium, with the highest yield (by isolate 49-22) being 5.5 g/l which corresponds to just 32.4 % conversion efficiency.

Several approaches were therefore investigated to attempt to improve IA yields from *A. terreus* fermentations. Firstly, several hydrolysate purification techniques were attempted. The use of activated charcoal improved RBDAH yields very slightly, although most treatments resulted in lower IA yield. Secondly, ultraviolet mutagenesis was attempted using two high IA producing isolates, 49-22 and 49-5. Several promising mutants were obtained including one showing a 3.5-fold increase over the parent. However, these high yields could not be replicated in subsequent experiments. Thirdly, attempts were made to induce the sexual cycle of *A. terreus* in order to generate genetically diverse offspring that might include progeny with improved IA production. A molecular diagnostic was used to determine mating type and several isolates of opposite *MAT* were crossed in all possible combinations on three media under various temperature and gaseous exchange conditions. Hyphal masses containing cleistothecia, asci and ascospores were produced from two weeks onwards, which varied in number according to conditions and crossing partners. Most isolates were of low fertility, but 49-40, 49-43 and 49-44 were identified as “super-maters”. Sorghum flour agar (SFA) produced approximately seven times more structures than other agar media assayed, the effect of temperature varied according to media, whilst allowing gas exchange resulted in more hyphal masses than sealing the plates. Although asci containing 4-8 ascospores were identified, difficulties were encountered in obtaining viable ascospore offspring. Only one putative recombinant offspring was obtained, as evidenced by molecular verification using RAPD-PCR and *MAT* markers. This isolate exhibited low IA yields in fermentations. Finally, fermentation optimisations were performed using the response surface methodology approach. A half-factorial screening experiment was used to select three fermentation factors then a central composite design (CCD) performed to obtain the optimum conditions. Optimum conditions for IA fermentation using RBDAH as a feedstock were found to be 30 °C, pH 4.0 and an *A. terreus* inoculum of  $1.0 \times 10^6$  spores/ml, resulting in 13.5 g/l IA and 28.3 % of theoretical maximum possible conversion, representing a 2.45-fold increase over non-optimised values obtained in initial time-point experiments. Higher pH and inoculum sizes, with lower temperatures favoured IA formation. Scale up to fermentation volumes of 200 ml and 500 ml was performed and these also generated even higher yields of 13.9 g/l and 16.3 g/l IA, corresponding to 49.6 % and 46.1 % of theoretical maximum respectively.

Overall, it was concluded that hydrolysates obtained from the wet-milling of sorghum bran can be used in the fermentative production of value-added chemicals with promising yields and efficiency, which warrants further research attention.

## Acknowledgements

My thanks are first and foremost to Almighty Allah (S.W.T) who has blessed me with the opportunity to, against so many odds, fulfil my dream of attaining this level of education. My gratitude also goes to the Tertiary Education Trust Fund (TETFund) of the Federal Government of Nigeria, Microbiology Department and the administration of the University of Ilorin for the intervention funds granted me to undertake this program.

I thank my initial supervisor Dr Chenyu Du for his support. I will also like to thank my supervisor Professor Paul Dyer for his guidance and assistance; I am privileged to have been supervised by him. My appreciation also goes to all the staff and students of the Bioenergy and Brewing Science building notably Jwan and Ivonne; the entire Food Sciences Division on the Sutton Bonington campus especially Drs. Ibbett and Linforth; and the Fungal Biology Group of the School of Life Sciences in University Park campus. I appreciate the support from the Nigerian community in the U.K., associating with them in various capacities made me less homesick. Thanks to numerous corporate sponsors and grantors for financial assistance which saw me through unanticipated tough times towards the end of my program. Thanks to everyone who supported me in any way (or didn't), I'm the better for it.

I will also like to thank the Badirus for their help over these years; to all my wonderful in-laws, particularly the Alh. Abdulganiyu Ahmad Al-Imams who were extra supportive of me in several ways (Mummy Basitu e seun mi o!). Thanks to my entire family for all their support during my long absence particularly when the kids returned without me. I am indeed very blessed. To my parents Alhaji and Alhaja A. Mustapha Dundu, I would not have been able to do any of this without you. Thanks for all your help Mummy, you are my rock, and to Daddy for all your sacrifices. Oloun a je ki e pe fun wa, ninu emi gigun ati alafia, amin.

Finally, I thank my young family for their support and encouragement. I took on a challenge that was enormous in several other ways besides academic, and you all stood by me without equivocation. Thank you Olowo ori Kikelomo Dr. Ibrahim Ahmed El-Imam, you are the best husband a woman could ask for; your support for me and my career is the reason this degree ever came to be in the first place. O seun o! I apologise to my babies Olayinka, Oyindamola, Olayiwola and my latest bundle of joy Oyewole for the disruptions to their lives and the family unit; Mum thanks and loves you beyond words. Thanks for putting up with it all.

Gaskiya, na gode wa Allah! Alhamdulillah.



## Abbreviations

Abbreviation	Meaning
ACM	<i>Aspergillus</i> complete media
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
b	Billion
bp	Base pair
benA	Beta tubulin primer
CAD	<i>cis</i> -aconitate decarboxylase
CCD	Central composite design
cm	Centimetre
CVS	Calibration verification standard
°C	Degrees Celsius
DAH	Dilute acid hydrolysate
DNA	Deoxyribonucleic acid
DNS	3,5-Dinitrosalicylic acid
dNTP	Deoxyribonucleotide triphosphate
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EH	Enzyme hydrolysate
EJ	Exajoule ( $10^{18}$ J)
g	Gram
GC	Gas chromatography
GHG	Greenhouse gas
HPLC	High performance liquid chromatography
h	Hour
IA	Itaconic acid
Kb	Kilobase
l	Litre
LAP	Laboratory analytical procedure
MC	Moisture content
MCA	Mixed Cereal Agar
mg	Milligram

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min	Minute
mM	Millimolar
MT	Metric tonnes
ng	Nanogram
OOA	Odlum's oatmeal agar
ODW	Oven dry weight
ORF	Open reading frame
OVT	One variable at a time
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PM	Phenotypic microarray
psi	Pounds per square inch
RAPD	Random amplified polymorphic DNA
RBDAH	Red bran dilute acid hydrolysate
RBEH	Red bran enzyme hydrolysate
RI	Refractive index
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSM	Response surface methodology
SB	Sorghum bran
SBH	Sorghum bran hydrolysate
SDS	Sodium dodecyl sulphate
sec	Second
SFA	Sorghum flour agar
TE	Tris-EDTA
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEA	Triethanolamine
µg	Microgram
µL	Microliter
v/v	Volume per volume
WBDAH	White bran dilute acid hydrolysate
WBEH	White bran enzyme hydrolysate

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wk	Week
w/v	Weight per volume

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# Table of Contents

Abstract.....	i
Acknowledgements.....	vi
Abbreviations.....	vii
Table of Contents.....	x
List of Figures.....	xv
List of Tables.....	xxiii
List of Appendices.....	xxvi
Chapter 1: General Introduction.....	1
1.1 Introduction.....	1
1.1.1 Fossil Resources: Versatile Sources of Energy and Chemicals.....	1
1.1.2 Trends in Global Fossil Resource Consumption.....	2
1.1.3 Impact of Current Fossil Resource Consumption Rates.....	3
1.1.4 Renewable Sources of Fuels and Chemicals.....	4
1.1.5 Challenges of First Generation Biofuels and Chemicals.....	4
1.1.6 Second Generation Biofuels and Chemicals.....	5
1.1.7 Challenges of the Second Generation Route.....	6
1.2 Background on Bioconversions.....	6
1.2.1 Fermentation.....	6
1.2.2 History of Fermentation.....	7
1.2.3 Fuels and Chemicals from Conventional Raw Materials.....	8
1.2.4 Sustainability.....	9
1.2.5 Factors Propelling Sustainability.....	11
1.2.6 Renewable Raw Materials - Biomass.....	12
1.2.7 Pretreatment of Biomass.....	22
1.2.8 Renewable Fuels and Chemicals.....	27
1.3 Biorefineries.....	30

1.4 Problem Statement .....	32
1.5 Aim and Objectives.....	32
Chapter 2: General Materials and Methods .....	34
2.1 Materials .....	34
2.1.1 Microorganisms .....	34
2.1.2 Sorghum Bran .....	35
2.1.3 Defined Ethanol Fermentation Medium .....	35
2.1.4 Defined Fungal Fermentation Medium.....	35
2.1.5 Other Chemical Solutions and Buffers .....	35
2.2 Methods.....	37
2.2.1 Wet-Milling of Sorghum Grains.....	37
2.2.2 Storage of Microorganisms.....	39
2.2.3 Preparation of Microorganism Cultures on Solid Media.....	39
2.2.4 Itaconic Acid Fermentation.....	40
2.2.5 Ethanol Fermentation.....	41
2.2.6 HPLC Analyses.....	42
2.2.7 Statistical Analyses .....	42
Chapter 3: Sorghum Bran Characterisation and Pretreatment.....	43
3.1 Introduction.....	43
3.1.1 Sorghum Bran Composition .....	43
3.1.2 Sorghum Bran Pretreatment.....	44
3.1.3 Fermentation Inhibitors.....	45
3.1.4 Aims .....	46
3.2 Materials and Methods.....	46
3.2.1 Materials .....	47
3.2.2 Methods.....	49
3.2.3 Generation of Hydrolysate .....	58
3.2.4 Inhibitory Compound Analysis.....	61

3.3 Results.....	61
3.3.1 Compositional Analysis.....	61
3.3.2 Other Compositional Analyses.....	68
3.3.3 Hydrolysates Generation.....	71
3.3.4 Sample Hydrolysates.....	80
3.3.5 Inhibitor Analysis.....	82
3.4 Discussion.....	84
3.4.1 Compositional Analyses For Summative Mass Closure.....	85
3.4.2 Other Compositional Analyses.....	88
3.4.3 Hydrolysate Generation.....	89
3.4.4 Sample Hydrolysates.....	93
3.4.5 Inhibitor Analysis.....	93
3.5 Summary And Conclusions.....	94
Chapter 4: Production of Value-Added Chemicals from Sorghum Bran.....	96
4.1: Introduction.....	96
4.1.1 Ethanol.....	98
4.1.2 Itaconic Acid.....	106
4.1.3 Aims.....	119
4.2 Materials and Methods.....	119
4.2.1 Ethanol Production.....	120
4.2.2 Itaconic Acid Production.....	125
4.3 Results.....	128
4.3.1 Ethanol Production.....	128
4.3.2 Itaconic Acid Production.....	138
4.4 Discussion.....	152
4.4.1 Ethanol Production.....	152
4.4.2 Itaconic Acid Production.....	157

4.5 Summary and Conclusions .....	163
Chapter 5: Hydrolysate Purification .....	164
5.1 Introduction.....	164
5.1.1 Physical Detoxification.....	165
5.1.2 Biological Detoxification.....	167
5.1.3 Chemical Detoxification .....	167
5.1.4 Aims.....	169
5.2 Materials and Methods.....	170
5.2.1 Materials .....	170
5.2.2 Methods.....	171
5.3 Results.....	178
5.3.1 Effect of Hydrolysate Detoxification on Hydrolysate Parameters .....	178
5.3.2 Intrinsic Inhibitors.....	180
5.3.3 Itaconic Acid Fermentation.....	187
5.4 Discussion.....	188
5.4.1 Effect of Detoxification on Hydrolysates and Hydrolysate Parameters .....	189
5.4.2 Intrinsic Inhibitors.....	192
5.4.3 Itaconic Acid Fermentation.....	196
5.4.4 Summary and Conclusions .....	197
Chapter 6: Strain Improvement and Optimisation of Itaconic Acid Fermentation Process ..	198
6.1 Introduction.....	198
6.1.1 Classical Strain Improvement By Mutation.....	199
6.1.2 Genetic Recombination By Classical Processes .....	201
6.1.3 Genetic Recombination by Cloning and Genetic Engineering.....	206
6.1.4 Optimisation of Fermentation .....	207
6.2 Aims.....	208
6.3 Materials And Methods.....	210
6.3.1 Media, Solutions and Strains .....	210

6.3.2 Methods.....	214
6.3.3 Mutagenesis .....	214
6.3.4 Sexual Crossing .....	216
6.3.5 Fermentation Optimisation .....	223
6.3.6 Optimisation Verification and Scale-Up.....	227
6.4 Results.....	227
6.4.1 Ultraviolet Mutagenesis .....	228
6.4.2 Sexual Crossing .....	234
6.4.3 Fermentation Optimisation .....	246
6.5 Discussion .....	256
6.5.1 Ultraviolet Mutagenesis .....	256
6.5.2 Sexual Crossing .....	259
6.5.3 Fermentation Optimisations.....	265
6.6 Summary and Conclusion .....	269
Chapter 7: General Discussion.....	271
7.2 Generation of Hydrolysates .....	272
7.3 Ethanol Production.....	273
7.4 Itaconic Acid Production .....	274
7.5 Hydrolysate Purification/Detoxification.....	275
7.6 Strain Improvement and Process Optimisation.....	275
7.7: Future work.....	279
7.8 Conclusion .....	280
References.....	281
Appendices.....	308



## List of Figures

<b>Figure 1.1</b> The consumption of various energy resources over time (Tverberg, 2012).....	2
<b>Figure 1.2:</b> Total energy consumption in 2013 classified according to energy source (Wikipedia, 2016). .....	3
<b>Figure 1.3:</b> The four interconnected domains of sustainability. ....	10
<b>Figure 1.4:</b> The Sustainable Development Goals (SDGs) of the United Nations (UN, 2016). .....	11
<b>Figure 1.5:</b> Different biomass sources which can be used to produce renewable fuels and chemicals (compiled by author).....	13
<b>Figure 1.6:</b> Simplified structure of lignocellulose .....	14
<b>Figure 1.7:</b> The two polymers that make up starch, amylose and amylopectin.....	17
<b>Figure 1.8:</b> Mature grain sorghum crops in the field. Source <a href="http://www.agric.wa.gov.au">www.agric.wa.gov.au</a> .....	19
<b>Figure 1.9:</b> The sorghum caryopsis (NRI, 1996). .....	19
<b>Figure 1.10:</b> Simple schematic showing how pretreatment breaks lignocellulosic biomass into its components (Clifford, 2015). .....	24
<b>Figure 1.11:</b> Wet milling and dry milling processes of ethanol production (AGMRC, 2016) .....	25
<b>Figure 1.12:</b> Enzymatic hydrolysis of starch to glucose ( <a href="http://www.biotek.com">www.biotek.com</a> ).....	26
<b>Figure 1.13:</b> A maize-based biorefinery (Yang <i>et al.</i> , 2013). .....	31
<b>Figure 2.1:</b> A typical petrol-operated Burr-plate mill for wet-milling sorghum for wet-milling of grains and pulses in Nigeria. ....	37
<b>Figure 2.2:</b> Typical process of sorghum bran preparation from the wet-milling method.....	38
The sun-dried bran is stored in sealed polythene bags at room temperature pending use.....	38
<b>Figure 3.1:</b> The Mettler-Toledo® (HR83) halogen moisture content analyser. ....	50
<b>Figure 3.2:</b> White sorghum bran samples. Left: As obtained after extraction from wet-milled sorghum grains and air-dried; Right: After milling with the ball mill.....	63
<b>Figure 3.3:</b> Composition of the milling fractions obtained by sieving milled sorghum bran.	63
<b>Figure 3.4:</b> Time course of sorghum bran hydrolysis using amylolytic enzymes to achieve residual starch conversion into glucose. Bran loading rate was 20% and the reaction was held at 50°C for 24 hr, and amount of glucose estimated by HPLC. Values are averages (n = 3), error bars are standard deviations (some error bars are too small to be visible). ....	72
<b>Figure 3.5:</b> Effect of two concentrations of sulphuric acid on conversion efficiency of WB into reducing sugars during pretreatment for 15 or 30 min at a 5% solids loading rate.	

Amounts of sugars released are expressed as a percentage of bran hydrolysed. Values are averages (n = 3), error bars are standard deviations. ....	75
<b>Figure 3.6:</b> Effect of two concentrations of nitric acid on conversion efficiency of WB into reducing sugars during pretreatment at 121°C for 15 and 30 min at a 5% solids loading rate. Amounts of sugars released are expressed as a percentage of bran hydrolysed. Values are averages (n = 3), error bars are standard deviations. ....	75
<b>Figure 3.7:</b> Effect of two concentrations of sulphuric acid on conversion efficiency of RB into reducing sugars during pretreatment for 15 and 30 min at a 5% solids loading rate. Amounts of sugars released are expressed as a percentage of bran hydrolysed. Values are averages (n = 3), error bars are standard deviations. ....	77
<b>Figure 3.8:</b> Effect of two concentrations of nitric acid on conversion efficiency of RB into reducing sugars during pretreatment for 15 and 30 minutes at a 5% solids loading rate. Amounts of sugars released are expressed as a percentage of bran hydrolysed. Values are averages (n = 3), error bars are standard deviations. ....	77
<b>Figure 3.9:</b> The effect of various WB or RB bran loading rates on bran conversion efficiency into reducing sugars during dilute (3 %) sulphuric acid hydrolysis at 121°C for 15 min. The green line represents the proportion of bran (%) converted to glucose during digestion at the different conditions while the blue line represents same for xylose. The green and blue bars show the actual concentration of glucose and xylose liberated (g/L) respectively. Values are averages (n = 3), error bars are standard deviations. ....	78
<b>Figure 3.10:</b> Left: Sample Hydrolysates showing the appearance of the four hydrolysates prepared from sorghum bran. WBEH = White bran enzyme hydrolysate, WBDHAH = White bran dilute acid hydrolysate, RBEH = Red bran enzyme hydrolysate and RBDHAH = Red bran dilute acid hydrolysate. Right: 25 ml of RBDHAH dispensed into 250 ml Erlenmeyer flasks.	81
<b>Figure 3.11:</b> Chromatograms of the four hydrolysates as obtained from HPLC using a Hi-Plex H <sup>+</sup> column at 45 °C with a mobile phase of 0.005N H <sub>2</sub> SO <sub>4</sub> at flow rate of 0.4 ml/min. Clockwise: WBDHAH; RBDHAH; WBEH; RBEH. Peaks indicate different sugars. Retention times (r.t. in min.): Glucose = 14.52 min; Xylose = 15.42; Maltose = 12.42; Arabinose = 16.68. Note: Peaks may show slight shifts in r.t. of less than ± 0.1 min. ....	82
<b>Figure 4.1:</b> Primary and secondary metabolite production in microorganisms .....	97
<b>Figure 4.2:</b> Structure of ethanol (www.commonswikimedia.org ) .....	98
<b>Figure 4.3:</b> World leading producers of bioethanol (AFDC, 2016).....	101
<b>Figure 4.4:</b> Various ethanol producing microorganisms. A: <i>S. cerevisiae</i> ; B: <i>Z. mobilis</i> ; C: <i>S. stipitis</i> ; D: <i>C. thermosaccharolyticum</i> E: <i>C. thermocellum</i> .....	102

(Image credits A: <a href="http://www.bbsrc.ac.uk">www.bbsrc.ac.uk</a> B: <a href="http://www.ejbiotechnology.info">www.ejbiotechnology.info</a> C: <a href="http://genome.jgi.doe.gov">genome.jgi.doe.gov</a> ; D: <a href="http://www.aem.asm.org">www.aem.asm.org</a> ; E: <a href="http://www.lookfordiagnosis.com">www.lookfordiagnosis.com</a> ) .....	102
<b>Figure 4.5:</b> Global biofuel production 2000-2010 (Eisentraut, 2011) .....	105
<b>Figure 4.6:</b> Trend concerning itaconic acid research publications on Web of Science (Ahmed El-Imam and Du, 2014) .....	107
<b>Figure 4.7:</b> Itaconic acid crystals .....	108
<b>Figure 4.8:</b> Structure of itaconic acid ( <a href="http://www.sigmaaldrich.com">www.sigmaaldrich.com</a> ).....	108
<b>Figure 4.9:</b> Simplified schematic of the biosynthesis of itaconic acid (adapted from Ahmed El-Imam and Du, 2014). .....	110
<b>Figure 4.10:</b> Itaconic acid producing microorganisms. A: <i>Candida albicans</i> ; B: Spores of <i>Ustilago maydis</i> . Picture credit: A: <a href="http://www.collections.daff.qld.gov.au">www.collections.daff.qld.gov.au</a> ; B: <a href="http://www.biotrans.uni.wroc.pl">www.biotrans.uni.wroc.pl</a> .....	112
<b>Figure 4.11:</b> Industrial applications of itaconic acid (Ahmed El-Imam and Du, 2014). .....	117
<b>Figure 4.12:</b> Arrangement employed in inoculation in the spot-plate screening for tolerance of eight yeast strains to various sorghum bran hydrolysates on SBHA.....	122
<b>Figure 4.13:</b> Image of the bespoke 96-well plates showing the arrangement used to evaluate the effect of the hydrolysates on the cellular respiration of the yeast strains. ....	123
<b>Figure 4.14:</b> Spot-plate screening tests showing typical pattern of eight species of yeast growing on agar plates prepared from four different types of sorghum bran hydrolysates and viewed under UV light with a GelDoc Imaging system (note NCYC 2592 is a <i>S. cerevisiae</i> strain). A and C: Aerobic incubation; B and D: Anaerobic incubation. Spots comprise yeast cells in dilutions of $OD_{600} = 1$ stock from L-R: $10^{-1} - 10^{-4}$ . WBEH = white bran enzyme hydrolysate; RBEH = red bran enzyme hydrolysate; WBDAH = white bran dilute acid hydrolysate; RBDAH = red bran dilute acid hydrolysate. ....	129
<b>Figure 4.15:</b> Phenotypic microarray analysis showing metabolic activity of 8 yeast strains, as judged by redox signal intensity, cultivated on four sorghum bran hydrolysates and incubated anaerobically in an Omnilog system over 72 hr. Hydrolysate labelling as in <b>Figure 4.14</b> ...	131
<b>Figure 4.16:</b> Weight loss trends for mini-fermentations of four sorghum bran hydrolysates and a glucose medium control by strains of three different yeast species. WBEH = white bran enzyme hydrolysate; RBEH = red bran enzyme hydrolysate; WBDAH = white bran dilute acid hydrolysate; RBDAH= red bran bran dilute acid hydrolysate; YPD = Yeast Peptone Dextrose medium. All values are average of triplicate measurements. ....	133
<b>Figure 4.17:</b> Ethanol yields from the fermentation of various types of sorghum bran hydrolysates and YPD (as a control medium) over 120 h . Values are the yields as a function	

(%) of the theoretical maximum possible (Section 4.2.1.4); the total (%) yields are a sum of yields of analytes presented. All values are average of triplicate measurements (error bars indicate standard deviations).....	135
<b>Figure 4.18:</b> Time course fermentation with <i>K. marxianus</i> on red bran dilute acid hydrolysate over 120 hr. Values are means of triplicate fermentations. Error bars represent the standard deviation of the means. ....	137
<b>Figure 4.19:</b> Counts of <i>K. marxianus</i> cells over the fermentation period (120 hr) observed microscopically after staining with methylene blue. Values are averages of triplicate counts (error bars are standard deviations).....	138
<b>Figure 4.20:</b> Typical chromatogram illustrating resolving of reference solutions of glucose and itaconic acid when performing HPLC using a Hi-Plex H <sup>+</sup> column at 45°C with a mobile phase of 0.005N H <sub>2</sub> SO <sub>4</sub> at flow rate of 0.4 ml/min. ....	139
<b>Figure 4.21:</b> Screening results of 46 <i>Aspergillus terreus</i> isolates investigated for itaconic acid production on defined glucose medium. Fermentations were performed at 37 °C and agitation rate of 200 rpm for 7 days. Fermentations were performed in biological duplicates and IA estimated in technical triplicates. Error bars are standard deviations.....	140
<b>Figure 4.22:</b> Distribution of itaconic acid production by <i>Aspergillus terreus</i> isolates (frequency represents number of strains) in defined medium. The yield values shown (g/l) represent itaconic acid yields plotted as bin centres of the histogram.....	141
<b>Figure 4.23:</b> Time course of itaconic acid production during fermentation of sorghum dilute acid hydrolysates (DAH) bran or control glucose medium by <i>Aspergillus terreus</i> isolates 49-5, 49-22 and 49-45. The fermentation conditions employed were: 37 °C, agitation/aeration rate 200 rpm, pH 3.1. Left: Itaconic acid yields. Right: sugar consumption trend showing residual glucose over time. Values are averages of triplicate fermentations and error bars represent standard deviations.....	143
<b>Figure 4.24:</b> Itaconic acid yields of the best-performing isolates of <i>A. terreus</i> obtained from the screening experiment after fermentation in sorghum bran hydrolysates for 4 days, or on control defined glucose media for 7 days. Values are (%) of the theoretical maximum possible and are averages of biological triplicates. Error bars indicate standard deviations.....	145
<b>Figure 4.25:</b> Time course of itaconic acid fermentation of glucose-enriched red sorghum bran dilute acid hydrolysate (RBDAH) (starting concentration of 120 g/l), by <i>Aspergillus terreus</i> at 37° C and 200 rpm for 9 days Left: Itaconic acid yields; Right: sugar consumption trend showing residual glucose over time.....	147

<b>Figure 4.26:</b> Itaconic acid yields from the fermentation of red bran hydrolysates and defined medium at 37 °C and 200 rpm. Yields are the (%) of the theoretical maximum possible and are average of triplicate experiments. Error bars indicate standard deviations. Values below the x axis indicate the media glucose content (note that the native RBDAH contains 51.6 g/l glucose without supplementation). .....	148
<b>Figure 4.27:</b> Mycelial mass produced by the <i>Aspergillus terreus</i> isolates in fermentation on red bran dilute acid hydrolysate (RBDAH) and defined medium at two different concentrations. The experiments were terminated on Day 7 except for the 51.6 g/l RBDAH, which was terminated on Day 4. Values presented are averages of biological triplicates. Error bars represent standard deviations. ....	149
<b>Figure 4.28:</b> Fed-batch fermentation of red bran dilute acid hydrolysate (RBDAH) with three <i>A. terreus</i> isolates over 11 days. Top Left: Itaconic acid production in RBDAH; Top Right: Glucose consumption in RBDAH fermentation; Bottom Left: Itaconic acid production in glucose defined medium; Bottom Right: Glucose consumption in defined medium. Arrows indicate point of glucose replenishment. ....	151
<b>Figure 5.1:</b> The appearances of the various red bran dilute acid hydrolysates after treatment by various detoxification methods. From left to right: regular (NaOH treated) RBDAH, lime neutralised RBDAH; overlimed RBDAH; activated carbon treated RBDAH; ethyl ethanoate extracted RBDAH; dowex 66 treated RBDAH; dowex 50WX2 treated RBDAH; dowex 50WX8 treated RBDAH; amberlite XAD16 treated RBDAH; and amberlite 2 treated RBDAH. ....	178
<b>Figure 5.2:</b> <i>Left:</i> The ultrafiltration set-up utilised in the RBDAH purification process. The left beaker contained the original RBDAH and the final retentate, while the permeate was collected in the left beaker. <i>Right:</i> The effect of ultrafiltration on the appearance of the fractions of RBDAH .....	179
<b>Figure 5.3:</b> Principal Component Analysis of the elemental composition of the RBDAH before and after phosphate removal treatment. ....	<b>Error! Bookmark not defined.</b>
<b>Figure 5.4:</b> Effect of various detoxification strategies on the yields of itaconic acid on red sorghum bran dilute acid hydrolysate (RBDAH) by <i>Aspergillus terreus</i> 49-22 at 37 °C and 200 rpm for 7 days. Yield values are the peak values obtained over the 7-day period and are presented here as a percentage of the theoretical maximum possible based on glucose consumed .....	187
<b>Figure 6.1:</b> A typical mutation and random selection process in microbial strain improvement (Parekh, 2009). ....	200

<b>Figure 6.2:</b> The sexual cycle in <i>Aspergillus</i> species (Khandelwal, 2016) .....	204
<b>Figure 6.4:</b> Itaconic acid concentration-gradient plate: the dark green lower layer indicates the 30 g/l IAPDA and the top white layer, regular PDA. ....	214
<b>Figure 6.5:</b> <i>Left:</i> Spheres of different colours represent opposite mating types; arrows indicate distance of 4-5 cm between aliquots of the spore suspensions. <i>Right:</i> A barrage formation that resulted at the point of interaction after several weeks of incubation.....	221
<b>Figure 6.6:</b> Itaconic acid tolerance in two strains of <i>Aspergillus terreus</i> grown on PDA plates with varying concentration of itaconic acid. Values are averages of 6 measurements and error bars represent standard deviations. ....	229
<b>Figure 6.7:</b> Kill curve showing the decline in population survival of two <i>Aspergillus terreus</i> isolates due to exposure to ultraviolet irradiation. The broken lines show the 5 % survival points for both strains. Values are averages of triplicate measurements and error bars represent standard deviations. ....	229
<b>Figure 6.8:</b> Cultures of parent and mutant progenies of <i>Aspergillus terreus</i> 49-5 and 49-22 on control PDA and 30 g/l IAPDA plates. <i>Top - Aspergillus terreus</i> 49-5, <i>Left to Right:</i> Parental strain on PDA; on IAPDA; Mutant 49-5 progeny on PDA; on IAPDA. <i>Bottom – Aspergillus terreus</i> 49-22, <i>Left to Right</i> Parental strain on PDA; on IAPDA; Mutant 49-22 progeny on PDA; on IAPDA. IA concentration increasing from top to bottom of plates.....	230
<b>Figure 6.9:</b> A custom 12-well box showing the appearance of M59-M70 at the end of the mutation screening fermentation. The white rings inside the wells are <i>Aspergillus terreus</i> growth. ....	231
<b>Figure 6.10:</b> Itaconic acid concentrations of highest-yielding mutants obtained from UV mutagenesis of two isolates of <i>Aspergillus terreus</i> after fermentation for seven days. Blue bars represent 49-5 mutant progeny, and red bars 49-22 mutant progeny; dashed line represents parent 49-5 yields (4.3 g/l) while continuous line represents parent 49-22 yields (4.7 g/l).....	232
<b>Figure 6.11:</b> Frequency histograms of distribution of itaconic acid yields by <i>Aspergillus terreus</i> UV mutants in RBDAH fermentation. Top: UV mutants derived from 49-5, n = 90; Bottom: UV mutants derived from 49-22, n=160. Bins separate IA yields into classes 0.5 g/L apart; Frequency shows number of progeny in each bin .....	233
<b>Figure 6.12:</b> Agarose gel (1%) of PCR amplification products of isolates of <i>Aspergillus terreus</i> showing mating type amplicons of isolates employed in sexual crosses. The 100bp molecular weight marker is denoted by M; lanes are labelled with the BDUN strain identification code; C1 and C2 are water controls for ATEM1 and ATEM2 respectively. ...	235

<b>Figure 6.13:</b> Sexual crosses set up between various <i>Aspergillus terreus</i> strains of opposite mating types. A: Appearance of a cross on SFA plate after five days; B: Cream-coloured hyphal masses formed at barrage zone on SFA plate; C: Fully mature SFA plate with several masses at barrage zone; D: Crosses of 49-5 on MCA showing formation of white mycelial film, the lower half of the plate has been peeled back to reveal spherical masses; E: Micrograph of the masses showing spherical bodies alongside cylindrical conidiospores aggregations; F: Exudate/liquid droplets found on the surface of 49-5 crosses; G: Hyphal aggregations with a flattened appearance from 49-22 x 49-5 cross; H: Sole pink mass resulting from 49-22 x 49-1 cross.	237
<b>Figure 6.14:</b> Effect of medium type on the formation of hyphal masses during sexual crossing under two conditions when plates were either sealed with Nescofilm, or were placed in an unsealed polythene bag. Media labelling as in <b>Table 6.10</b> .	241
<b>Figure 6.15:</b> Effect of air exchange on the formation of hyphal masses during sexual crossing on the three media. Media labelling as in <b>Table 6.10</b> .	241
<b>Figure 6.16:</b> Effect of incubation temperature on the formation of hyphal masses during sexual crossing on three media. Media labelling as in <b>Table 6.10</b> .	243
<b>Figure 6.17:</b> Photomicrographs of sexual structures of <i>Aspergillus terreus</i> taken after 12 weeks incubation. A: Hyphal mass under stereomicroscope; B: Mass cleaned of hyphal material on tap water agar to reveal putative cleistothecia; C: Intact mature cleistothecium (49-40 x 49-44 on SFA); D: Ruptured cleistothecium (49-40 x 49-44 on SFA); E: Intact mature ascus with eight ascospores (49-22 x 49-44 on SFA); F: Leaky ascus with six ascospores and within and outside the ascus (49-43 x 49-44 on OOA); G: Developing ascus with few visible ascospores (49-22 x 49-44 on SFA); H: Immature ascus (45 x 44 on OOA).	244
<b>Figure 6.19:</b> Evidence of meiotic recombination in progeny from sexual cross between parental strains 49-43 and 49-44 of <i>Aspergillus terreus</i> as detected using the AMIII primer. Headings: MM = 100 bp Molecular Marker; Alphabets denote putative ascospore isolates; numbers denote parent strain numbers following the common ID “49-”. C = water control. Blue bars = <i>MAT I-1</i> , pink bars = <i>MAT I-2</i> mating types. Arrow points at diagnostic band in progeny “T” which is a <i>MAT I-1</i> type but exhibits a band from the <i>MAT I-2</i> parent.	245
<b>Figure 6.20:</b> Pareto chart showing the variables in decreasing order of importance with the length of each bar being proportional to its standardised effect. Bars extending beyond the vertical blue line correspond to effects which are statistically significant at the 95.0% confidence level.	249

<b>Figure 6.21:</b> Main Effects Plot showing the estimated itaconic acid as a function of the various experimental factors. ....	249
<b>Figure 6.22:</b> Main Effects Plot showing the estimated itaconic acid as a function of the various experimental factors. ....	252
<b>Figure 6.23:</b> Pareto chart showing the estimated effects in decreasing order of importance. Values extending beyond the vertical blue line are statistically significant at the 95.0% confidence level. ....	252
<b>Figure 6.24:</b> Response surface mesh showing effect of the interactions of the various parameters on predicted IA yields. ....	254
<b>Figure 7.1:</b> Flowchart summarising the bioconversion of sorghum bran into value-added chemicals.....	278



## List of Tables

<b>Table 1.1:</b> Composition of some biomass materials. ....	16
<b>Table 2.1:</b> Preparation of 1L of standard solutions of simple sugars and itaconic acid.....	36
<b>Table 3.1:</b> Preparation of samples for lignin determination.....	55
<b>Table 3.2:</b> Moisture content values of the sorghum brans as determined with a halogen moisture analyser. Values are presented as means of triplicate analyses with the standard deviations .....	62
<b>Table 3.3:</b> The ash content as % w/w of both sorghum brans. Values are presented as means of triplicate analyses with the standard deviations. ....	64
<b>Table 3.4:</b> Carbohydrate content of the sorghum brans expressed (% w/w) as the detectable sugars. Values are presented as means of triplicate analyses with the standard deviations. ...	65
<b>Table 3.5:</b> The lignin content of both sorghum brans (% w/w) as determined by the acetyl bromide method. Values are presented as means of triplicate analyses with their standard deviations. ....	66
<b>Table 3.6:</b> Protein content (% w/w) of both sorghum brans as determined by gas chromatography. Values are presented as means of triplicate analyses with the standard deviations. ....	67
<b>Table 3.7:</b> Lipid content as % w/w of both sorghum brans as determined by gas chromatography. Values are presented as means of triplicate analyses with the standard deviations. ....	67
<b>Table 3.8:</b> Summative mass closure of the white and red sorghum brans employed in bioconversion processes in this work. ....	68
<b>Table 3.9:</b> Starch content (% w/w) of the sorghum brans as determined following complete amylase and amyloglucosidase digestion. Values are presented as means of triplicate analyses with the standard deviations.....	69
<b>Table 3.10:</b> Structural carbohydrate content (% w/w) of the sorghum brans. Values are presented as means of triplicate analyses with the standard deviations.....	70
<b>Table 3.11:</b> Reducing sugar content (% w/w) of sorghum brans determined by the dinitrosalicylic acid (DNS) method. Values are presented as means of triplicate analyses with the standard deviations.....	71
<b>Table 3.12:</b> Sugars released in dilute acid optimisation experiments. Values are averages of triplicate observations with the standard deviations. ....	73
<b>Table 3.13:</b> Multiple Range Tests for Conversion to Glucose by SL .....	79

<b>Table 3.14:</b> Glucose contents of various sorghum bran hydrolysates.....	81
<b>Table 3.15:</b> Amounts of various fermentation inhibitors found in a batch of the four hydrolysates. Values are averages of triplicate measurements and the standard deviations. ..	84
<b>Table 3.16:</b> Concentrations of common inhibitory compounds present in various lignocellulosic hydrolysates.....	94
<b>Table 4.2:</b> Multiple range test for ethanol yield (%) comparing the performance of the various media (data pooled for all different yeast strains).....	136
<b>Table 4.3:</b> Multiple Range Tests for itaconic acid by strain .....	151
<b>Table 5.1:</b> Effect of the various detoxification treatments on glucose content of red bran dilute acid hydrolysate. ....	180
<b>Table 5.2:</b> Total phenolic compounds in the various hydrolysates as determined by the Folin-Ciocalteu test and phenolic removal (combined <i>p-coumaric</i> and ferulic acid values). ....	182
<b>Table 5.3:</b> Condensed tannin (CT) content of the purified hydrolysates as determined by the Vanillin-HCl method. ....	184
<b>Table 5.4:</b> Multi-element analysis performed by Inductively Coupled Plasma Mass Spectrometry (ICPMS) using a Thermo-Fisher iCAP-Q system equipped with collision cell technology with energy discrimination (CCTED).....	186
<b>Table 5.5:</b> Table of component weights from the PCA of the elemental composition of the RBDHAH and PhosGuard <sup>TM</sup> treated RBDHAH .....	<b>Error! Bookmark not defined.</b>
<b>Table 6.1:</b> Constraints determining the design of industrial fermentation processes [Kuenzi and Auden, 1983 (cited in Smith, 2012)].....	209
<b>Table 6.2:</b> Preparation of various concentrations of itaconic acid potato dextrose agar (IAPDA) plates .....	213
<b>Table 6.3:</b> Amplification primers used in the species verification analysis for isolates of <i>Aspergillus terreus</i> . ....	218
<b>Table 6.4:</b> Amplification primers for mating-type determination in the <i>MAT</i> region of <i>Aspergillus terreus</i> .....	219
<b>Table 6.5:</b> List of primers used in RAPD-PCR for the investigation of genetic recombination. ....	223
<b>Table 6.6:</b> Variables and the levels employed in a half-factorial design for itaconic acid fermentation. ....	225
<b>Table 6.7:</b> Half factorial design used to screen factors for itaconic acid fermentation using <i>Aspergillus terreus</i> . ....	226

<b>Table 6.8:</b> Base design for the central composite design employed for the optimisation of the itaconic acid fermentation in RBDAH by <i>Aspergillus terreus</i> .	227
<b>Table 6.9:</b> Itaconic acid yields after 7 days fermentation from shake-flask experiments with high-yielding mutants of <i>Aspergillus terreus</i> obtained from UV screening experiments	233
<b>Table 6.10:</b> The effect of three different media, two incubation temperatures and sealing on the total number of sexual reproductive structures produced by isolates of <i>Aspergillus terreus</i> after 12 weeks incubation. <b>Figures</b> indicate numbers of hyphal masses observed, and are colour coded (red= no hyphal masses; purple = 1-25 masses; dark purple = 25-100 masses; grey= 101-150 masses; green = 151-250 masses).	239
<b>Table 6.11:</b> Results of half-factorial design screening of factors important in itaconic acid production by <i>Aspergillus terreus</i> 49-22 fermentation on RBDAH.	248
<b>Table 6.12:</b> Central composite experimental design for itaconic acid fermentation and the results obtained	251
<b>Table 6.13:</b> Analysis of variance for optimisation of itaconic acid production on RBDAH by RSM	253
<b>Table 6.14:</b> Conditions for response optimisation of itaconic acid fermentation.	254
<b>Table 6.15:</b> Itaconic acid yields from the optimum conditions selected from the CCD experiments and the optimum conditions predicted by the model for fermentation in RBDAH	255
<b>Table 6.16:</b> Scale-up fermentation experiments for itaconic acid production from RBDAH. Values are averages of triplicates, with the standard deviations.	256
<b>Appendix 8:</b> Publications stemming from this study	320

## List of Appendices

<b>Appendix 1:</b> Strains of <i>Aspergillus terreus</i> employed in this work and details of their source and mating types.....	296
<b>Appendix 2:</b> Yeast species/strains employed in ethanol fermentations.....	298
<b>Appendix 3:</b> Itaconic acid yields obtained from the fermentation of five <i>Aspergillus terreus</i> strains on RBDAH and defined glucose media of different concentrations. Values are averages of triplicates with the standard deviation.....	298
<b>Appendix 4:</b> Multiple Range Tests for itaconic acid yield (%) by hydrolysate purification type. Method: 95.0 percent LSD.....	299
<b>Appendix 5:</b> Schedule of crosses .....	301
<b>Appendix 6:</b> Itaconic acid yields of all strains obtained from the mutation of strains 49-5 and 49-22.....	305
<b>Appendix 7:</b> Estimation results for itaconic acid from RSM .....	307
<b>Appendix 8:</b> Publications stemming from this study.....	308

# **Chapter 1: General Introduction**

## **1.1 Introduction**

Value-added products as referred to in this thesis are simply products obtained from petroleum or biological processes which are of higher value than the raw material from which they are produced. Work in the current study is involved with assessing whether sorghum bran can be used to produce particular higher value products via fungal fermentation. By way of introduction, the need for novel approaches to produce value-added products will first be presented, before more detailed discussion of the exploitation of fungal fermentation and possible use of sorghum bran as a substrate. There is a focus on the relevance to Nigeria, the sponsoring country of work in the present thesis.

### **1.1.1 Fossil Resources: Versatile Sources of Energy and Chemicals**

Almost all activities of modern life involve the use of products and fuel energy derived from fossil sources. There are three main fossil resources from which global carbon demand for fuel and chemicals are met, namely: petroleum, coal and natural gas.

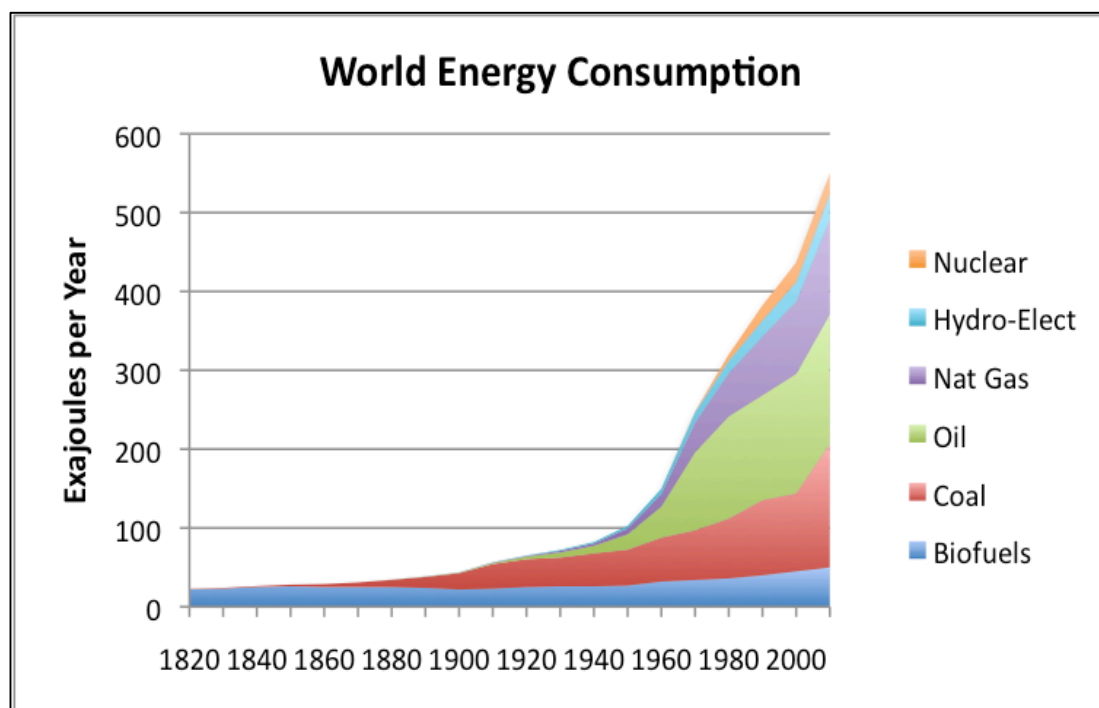
Petroleum is the principal source and it is separated into various constituent fuels, chemicals and other products through several refining processes and operations in refineries. The most important petroleum refining products by demand are transportation fuels rich in chemical energy and comprising petrol used to power automobiles, kerosene used in jets, and dyes (Gary *et al.*, 2007). The petroleum-based chemical products, also known as petrochemicals or petroleum distillates, are categorised into three classes: the olefins, the aromatics and syngas. Olefins including ethylene, propylene and butadiene are the largest volume petrochemicals produced worldwide and are almost entirely used as building blocks in the production of textiles, plastics, rubber etc. (Dreyer, 2007). The aromatics such as benzene and xylene are produced from the naphtha component of petroleum and used in the manufacture of detergents and polyurethanes, while syngas is used for the production of fertilizers and chemicals (Dreyer, 2007; Gary *et al.*, 2007).

Coal is a sedimentary rock formed in a process called coalification (Fanchi, 2013). It was the main fuel used during the industrial revolution but is now mainly used as a fuel in power

plants and to produce coke for metallurgy. It is also gasified to produce syngas, or liquefied to obtain synthetic fuels.

### 1.1.2 Trends in Global Fossil Resource Consumption

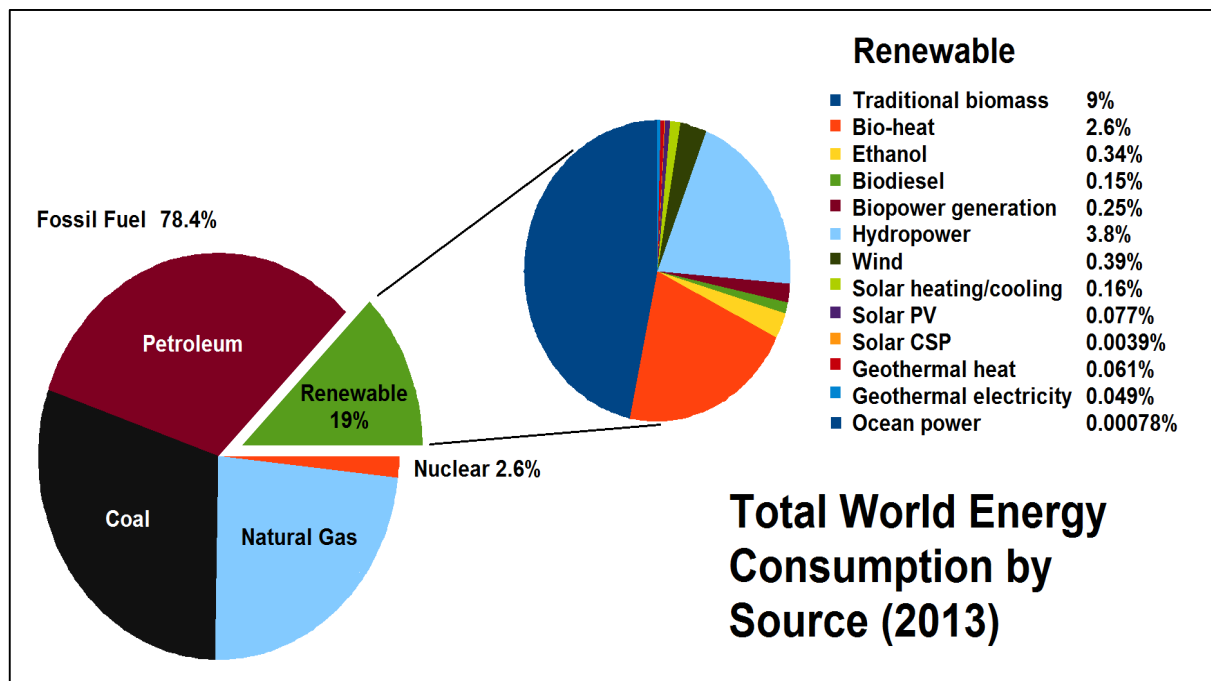
Food was the first source of energy for humans, then for millennia human energy needs have been met in addition by fossil fuels. Petroleum was collected from seepages around 3,000 BC in the Middle East while coal was the first fossil fuel to be used widely (Fanchi, 2013). Human energy needs and consumption patterns changed as society evolved as seen in **Figure 1.1**. As the population and prosperity increased, global energy consumption increased from an estimated 16 EJ in 1860 to 403 EJ by 1990 (Klass, 1998).



**Figure 1.1** The consumption of various energy resources over time (Tverberg, 2012).

Within this time, the world's population has doubled sequentially thrice while fossil fuel consumption has doubled six times (Klass, 1998). This indicates a considerable increase in energy consumption over time with fossil sources contributing almost 80% of this energy (**Figure 1.2**). Fossil sources have also been a key source of other chemicals; about 10% of petroleum uses goes to non-fuel uses such as production of chemicals and lubricants (Lucia *et*

*al.*, 2006). About 4% of propylene produced from the olefin fraction of petroleum refining is used in the production of acrylic acid, which is a key industrial chemical used manufacture of diapers, paints etc. (Thammanayakatip, 2015). It is thus evident that fossil resources play a key role in our daily lives.



**Figure 1.2:** Total energy consumption in 2013 classified according to energy source (Wikipedia, 2016).

### 1.1.3 Impact of Current Fossil Resource Consumption Rates

For various reasons the current trend in the consumption of fossil resources is not sustainable. Firstly, widespread petroleum extraction is depleting finite fossil fuels reserves, and given the extensive periods (millions of years) required to replenish them, they are thus described as non-renewable resources which will eventually run out (Klass, 1998). Another impact is that fossil fuel combustion has been linked with undesirable climate and environmental changes. For instance they have been definitively shown to increase the amounts of greenhouse gases (GHGs) thus raising the earth's temperature (Wuebbles and Jain, 2001). Fossil fuel extraction also has a negative climatic footprint as seen, for instance, in Nigeria where gas flaring and the oil spills have ruined the livelihoods of entire communities. This has resulted in among other cases, a King from Nigeria's oil-rich Niger-Delta suing Royal Dutch Shell in a British

High Court for oil spills that have devastated communities for decades (Mustoe, 2016). This relationship between increasing fossil resource use, declining reserves and climatic and environmental problems indicates that an energy crisis is imminent and near-inevitable unless measures are put in place.

#### **1.1.4 Renewable Sources of Fuels and Chemicals**

Various steps have been taken to minimize the rate and impact of climate change, improve sustainability and reduce contributions to the carbon cycle. A key solution to this is to minimise the release of anthropogenic GHGs found into the atmosphere. To this end, participating countries at the 1992 Rio de Janeiro Earth Summit agreed to the Climate Change Convention which eventually led to the Kyoto Protocol in 1997 and in 2015 the Paris Agreement, which all aim to reduce GHGs emission (Fanchi, 2013) among other commitments.

Consequently, renewable carbon sources of plant origin, known as biomass, have been extensively investigated for the production of bio-based fuels and chemicals, known as first generation (1<sup>st</sup> gen) biofuels and chemicals or 1<sup>st</sup> gen biocommodities which are either same as, or completely new products which can replace, the fossil-derived products. Biomass is the most important source of renewable energy today (Sims *et al.*, 2008). Several technologies exist at advanced stages for biomass transformation such as fermentation, anaerobic digestion and valorisation and have generated a wide array of biocommodities. The most common example is the fermentative conversion of carbohydrates in foods like beet, maize, sugar cane sweet sorghum etc. into ethanol for use as a transportation fuel. This process based on simple food sugars is well established now but has some limitations.

#### **1.1.5 Challenges of First Generation Biofuels and Chemicals**

There are inherent challenges in the use of potential foodstuffs for the production of transport fuels or chemicals i.e. the first generation route. Competition in land, pesticide, labour and water use between crops for food and biofuel crops can result in changes in land use. Life cycle assessments have also shown that after incorporating emissions from transport and production, the carbon footprint of these 1<sup>st</sup> gen processes were close to those of fossil fuels (Sims *et al.*, 2008). However, most notably, the 1<sup>st</sup> gen biofuels created a demand on an



already stretched food supply system as biofuel mandates meant crops were increasingly diverted away from food uses to produce fuels, causing some food prices to rise up to 40% in one year (Tenenbaum, 2008). The food vs fuel debate thus became a major morality question.

### **1.1.6 Second Generation Biofuels and Chemicals**

Several of the challenges associated with 1<sup>st</sup> gen processes are, to a great extent, mitigated by the increasing use of non-food, renewable carbon sources for the production of value-added chemicals. This is particularly true for the ‘food vs fuel’ problem as forestry wastes, agricultural wastes, and energy crops such as *Miscanthus sp.*, can be used to produce biofuels and chemicals. These feedstocks are usually called lignocellulosic wastes as they are composed of cellulose, hemicellulose and lignin. However, there are also food wastes which contain residual starch from processing such as wheat bran and sorghum bran that may also be exploited. Lignocellulosic wastes usually require some pretreatments to disrupt the recalcitrant lignocellulose structure, prior to enzymatic hydrolysis into simple sugars that are then fermented by microorganisms (Zheng et al., 2009).

Agricultural feedstocks/residues are potentially the cheapest biomass feedstocks available in significant quantities (Sims *et al.*, 2008). Several different agricultural wastes accumulate in developing countries and their use for production of higher-value products is a much needed value-addition for locals in addition to ridding the environment of organic waste. Sugarcane bagasse and leaves accumulate worldwide to the tune of about 279 million metric tonnes annually (Chandel *et al.*, 2012). Meanwhile, Nigeria is a leading producer of sorghum, with the bran residue of its processing accumulating in the environment or fed to animals as low-value feed.

These 2<sup>nd</sup> gen technologies are currently at the demonstration and pilot plant stages and still have some technical hurdles to overcome.

Currently, so-called ‘3<sup>rd</sup> generation’ biofuels are also being developed from microalgae which are unicellular microorganisms found in fresh water and marine environments. Microalgae are generally more efficient converters of solar energy than higher plants, and because they grow in aqueous media, they have better access to nutrients (Alam *et al.*, 2015; Kostas *et al.*, 2016).

### **1.1.7 Challenges of the Second Generation Route**

In spite of their superior characteristics relative to 1<sup>st</sup> gen bio-products, and fossil sources of high-value chemicals, the economic feasibility of using the 2<sup>nd</sup> gen route to produce biocommodities from lignocellulose sources depends largely on the raw material, pretreatment method and capacity of the fermenting microorganisms to convert the sugars efficiently while tolerating fermentation inhibitors where present. The main cost incurred is from the conversion processes required to breach the plant biomass recalcitrance. Consequently, the technology pathways still need to be improved upon and costs reduced (Sims *et al.*, 2008). The greatest challenge towards the widespread development and uptake of 2<sup>nd</sup> gen products is the nascent stage of these technologies.

However, the utilisation of residual starch in starch-based renewable biomass may be easier as they have all the advantages of the lignocellulosic biomass without the challenge of recalcitrance. Moreover, it is possible to further degrade the cellulosic content of the biomass after the starch content has been utilised, to maximise the potential of this resource.

## **1.2 Background on Bioconversions**

There are two main conversion routes for the production of bio-commodities from plant biomass, viz: biochemical and thermochemical routes, with several variations of these routes under evaluation (Sims *et al.*, 2008). A review of the current literature on the biochemical conversion of biomass into ethanol and itaconic acid will now be presented, to provide a general background for the research work in the current thesis.

### **1.2.1 Fermentation**

The term fermentation comes from a Latin word *fermentum* which means “to ferment” (Chojnacka, 2010). It is used in biochemistry to denote an anaerobic (or facultatively anaerobic) process in which reduced organic compounds, usually acids, are formed.

Historically fermentation products were only foods or drinks. However, nowadays, as in this project, it is used to refer to any metabolic process that utilizes a chemical change induced by an organism or its enzymes, in which carbohydrates and other compounds are partially oxidised to produce a specified product (Chojnacka, 2010). Industrial fermentation is thus a

key process whereby microorganisms (bacteria, yeasts and fungi) are specifically cultivated to convert basic raw materials such as sugars and oils into an almost unlimited range of substances that are useful to man (Soetaert and Vandamme, 2010). It is a key component of industrial (“White”) biotechnology, which is defined by Aftalion (2010) as “the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services”.

### **1.2.2 History of Fermentation**

Food fermentation has been practiced for millennia in ancient Egypt, China and other parts of the world, traditionally employed in the preservation of foods and involved in production of bread and alcoholic beverages (Soccol *et al.*, 2013). The Sumerians described the technique of brewing 6,000 years ago in the oldest discovered written records while 3,500 years ago soya fermentation was practiced in China (Buchholz and Collins, 2013). Fermentation was also employed in the production of other foods in Asia such as the production of koumiss to preserve milk, and the saccharification of rice by moulds in the "koji process".

In the 14<sup>th</sup> century ethanol distillation from grains was practiced, and then in the mid-19<sup>th</sup> century the fermentative production of both alcohols and chemicals such as organic acids was reported (Vasic-Racki, 2008). In 1857 Louis Pasteur demonstrated that lactic acid fermentation was carried out by living cells-yeasts, leading to the foundation of the scientific discipline of microbiology. Edward Buchner demonstrated in 1897 that cell-free filtrates of yeast cultures also carried out fermentation of sucrose into ethanol, thus initiating a biochemical paradigm. It was concluded that living microorganisms produced enzymes which degraded sugars into alcohols (in this case zymase). Buchner’s work stimulated the molecular approach to fermentation and proved that enzymes were the chemical foundation of bioconversions (Buchholz and Collins, 2013).

In 1923 Pfizer began producing citric acid from *Aspergillus niger* (Buchholz and Collins, 2013). Then in 1928 Alexander Fleming demonstrated the production of penicillin from *Pencillium chrysogenum*, while the research group of Florey, Chain and Heatley in Oxford (UK) later produced it in surface culture and tested its efficacy in 1940. Following intense international and interdisciplinary collaboration with groups in the USA, which helped raise yields, penicillin production became increasingly successful (Buchholz and Collins, 2013) with prices crashing from about \$25,000 per gram to \$30 per kg (Aftalion, 2010). After the

success of penicillin production, fermentation technology continued to advance and by the 1950s, large scale production of other antibiotics and high-value chemicals became widespread. Also in the 1950s, the structure of DNA and composition of RNA were elucidated thus giving rise to recombinant DNA technology which enables productions of some enzymes at levels 100 times higher than in native hosts (Singhania *et al.*, 2010). Subsequently, with the development of modern tools of molecular biology and genetic engineering, fermentation technology has improved considerably (Soccol *et al.*, 2013). More recently, fermentation has been widely utilised in the production of several high value products non-food products such as antibiotics, recombinant therapeutic products, fuels and chemicals.

### **1.2.3 Fuels and Chemicals from Conventional Raw Materials**

High-value products as referred to in this thesis are simply products obtained from petroleum or biological processes which are of higher value than the raw material from which they are produced.

The major raw materials conventionally used in the generation of energy are fossil fuels which are the decomposition products of plants and animals. There are three major forms of fossil fuels namely coal, petroleum and natural gas. Others include bitumen and oil shale (Gary *et al.*, 2007) even though these are currently not as economically important. The production of chemicals from these fossil raw materials is usually as a by-product of refining fossil fuels to produce fossil energy fuels.

**Coal:** Coal was the first fossil fuel to be used by man. Coal is a brown to black solid which is combusted for the production of electricity and heat and in the metal refining processes. Prior to the 1950s, particularly at the beginning of the industrial revolution, coal was also a major source of organic chemical feedstocks. However, in recent decades its use has been mainly limited to the generation of electricity by its combustion in power plants. It is used to produce aromatic speciality chemicals, but other materials currently produced from coal include metallurgical coke, activated carbons, etc. Unfortunately since the coal deposits of the world are finite it will eventually be depleted.

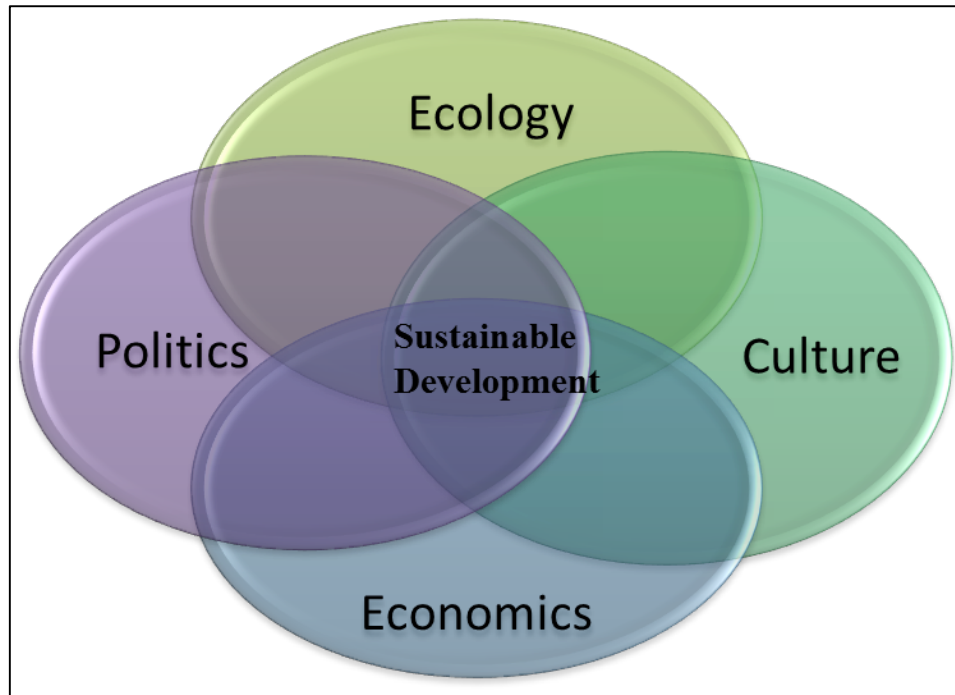
**Petroleum:** Since the mid-nineteenth century, petroleum has been the single most important source of fuels and chemicals for domestic and industrial uses. It is a dark, naturally

occurring viscous liquid which is formed from the decomposition of large amounts of dead life forms, mainly algae and planktons, underneath many layers found in geologic formations deep within the earth where they have been subjected to intensely high pressure and temperature conditions over thousands of years. Petroleum comprises mainly of a broad mix of various paraffin hydrocarbons and aromatics which are readily converted to fuels such as petrol, diesel and liquefied petroleum gas by refining (Dreyer, 2007). Although fossil fuels are still being created, the geological rate of formation is infinitesimally low compared to the rate of extraction and consumption (Gupta and Demirbas, 2010). The global proven crude oil deposit was put at 1,492.9 billion barrels at the end of 2014 (OPEC, 2014). Given concerns about peak oil production having already been reached, and a decline in production rate (Hubbert, 1956; Aleklett *et al.*, 2010), there are bleak forecasts of inevitable decline in future petroleum availability, and difficulties if global energy habits are not immediately changed.

**Natural gas:** Natural gas is a mixture of gaseous hydrocarbons comprising primarily methane (up to 90%), other alkanes and trace amounts of other gases. It is found associated with petroleum deposits. It is considered the cleanest fossil fuel because of its chemical simplicity (Gupta and Demirbas, 2010), which also accounts for its low energy density. It is used for heating, cooking, electricity generation, as a fuel and as a chemical feedstock in the manufacture of plastics, fertilizers and other important organic chemicals (Chandra, 2006).

#### **1.2.4 Sustainability**

Historically, the word “sustainability” was used in forestry to mean never felling more trees than the forest yields in new growth. In general terms it refers to the endurance of systems and processes. The term was first used in its modern connotation in the Brundtland report where it was defined as development that meets the needs of the present without compromising the ability of future generations to meet their own needs (Brundtland, 1987). At the core of sustainability is sustainable development which comprises the four interconnected domains of ecology or environmental protection, economic development, political participation and cultural diversity (Henkel, 2015) as depicted in **Figure 1.3** below.



**Figure 1.3:** The four interconnected domains of sustainability.

The four domains are split into 17 sustainable development goals (SDGs) which itemise targets and topics aimed at achieving the objective of “Transforming Our World: The 2030 Agenda For Sustainable development” (UN, 2016).

The use of all fossil fuels for energy generation or chemicals production releases anthropogenic (man-made) carbon dioxide which is a GHG into the environment. This has unfortunately led to the greenhouse effect, which is linked to the ability of atmospheric gases such as carbon dioxide and methane to trap some solar energy reemitted by the planet, with the rate of GHG release contributing to global warming (Wuebbles and Jain, 2001). This will directly hinder the achievement of several of the SDG aims including SDGs 3, 7, 11, 12 and 13 (**Figure 1.4**).



**Figure 1.4:** The Sustainable Development Goals (SDGs) of the United Nations (UN, 2016).

### 1.2.5 Factors Propelling Sustainability

The continued release of GHGs is not sustainable so to tackle this problem, the United Nations Framework Convention on Climate Change (otherwise called “The Kyoto Protocol”) came into force in 2005. It set binding obligations on industrialised countries to reduce greenhouse gas (GHG) emissions and encouraged developing countries to do the same (UNFCCC, 1997).

Key factors influencing the shift towards sustainability are

- The expectation that petroleum, being a finite resource, will eventually run out hence the need for a suitable alternative
- Industrialised nations now have to respect key criteria in the Kyoto treaty thus causing a change in the perception of resource use and energy consumption

- Prior to the fall in crude oil prices in 2015, the ever-rising price of petroleum led to a desire by most countries to diversify their energy sources; the rebound in prices has meant that renewable energy is still a priority
- Growing environmental awareness and societal concerns resulting in stricter environmental regulations aimed at minimizing the emission of greenhouse gases
- Rapidly increasing human populations accompanied by intensive agriculture resulting in the accumulation of rich waste biomass which are highly amenable to biological degradation
- The potential for achieving self-sufficiency in valuable chemicals can hasten a country's economic growth, especially in developing countries, many of which have abundant biomass and agricultural resources that are underutilized
- The need to encourage rural- and agriculture-based development (Bauen *et al.*, 2009).

The combination of these and many other local and regional factors have resulted in a steady shift towards the production of energy, chemicals and materials from renewable rather than fossilized resources. It is expected that the global economy will benefit from a change from profit-oriented to sustainable processes.

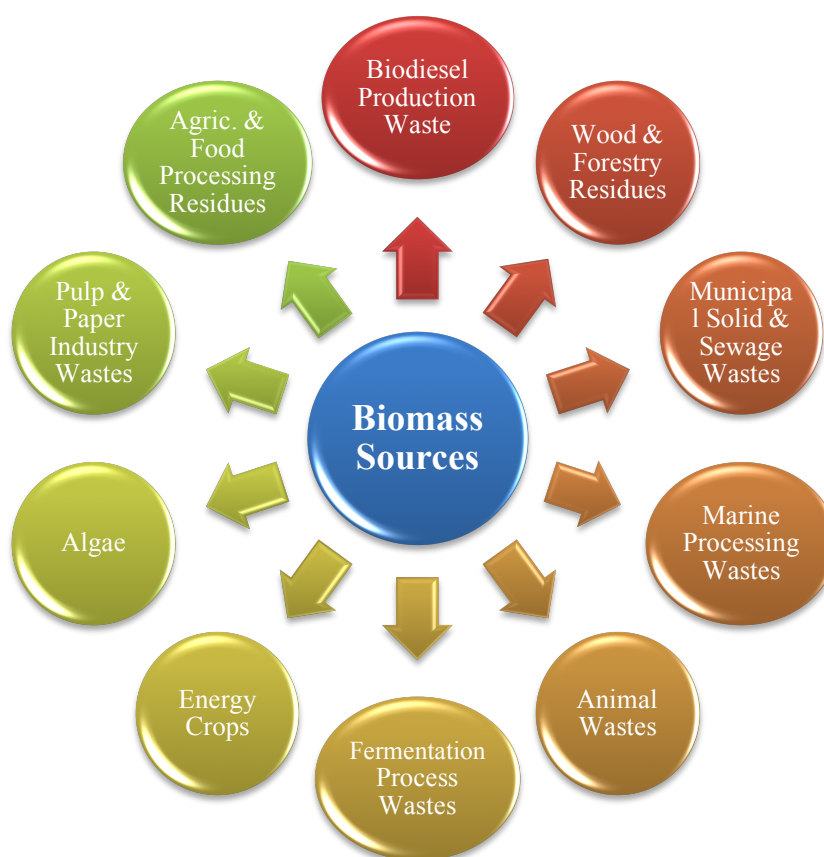
As the major determinant of a sustainable chemical industry requires a shift away from oil dependence, energy and chemical industries are looking into renewable sources and are also investing in research and the development of new technologies.

#### **1.2.6 Renewable Raw Materials - Biomass**

Renewable raw materials are defined as resources that have a natural rate of availability and yield a continual flow of services which may be consumed in any time period without endangering future consumption possibilities as long as current use does not exceed net renewal during the period under consideration (EIONET, 2016). There are several types of renewable raw materials including solar energy, wind and nuclear energy. Biomass is a renewable raw material and usually refers to the non-food part of plants. Biomass can have several definitions. However, in general, it refers to any organic material, derived from recently living organisms including animal manure containing mainly hydrogen, oxygen, nitrogen and trace amounts of other elements (Brar *et al.*, 2013). There is an abundance of



plant biomass and it is very heterogeneous depending on the source (**Figure 1.5**) with the annual global production estimated at over 100 trillion kilograms.

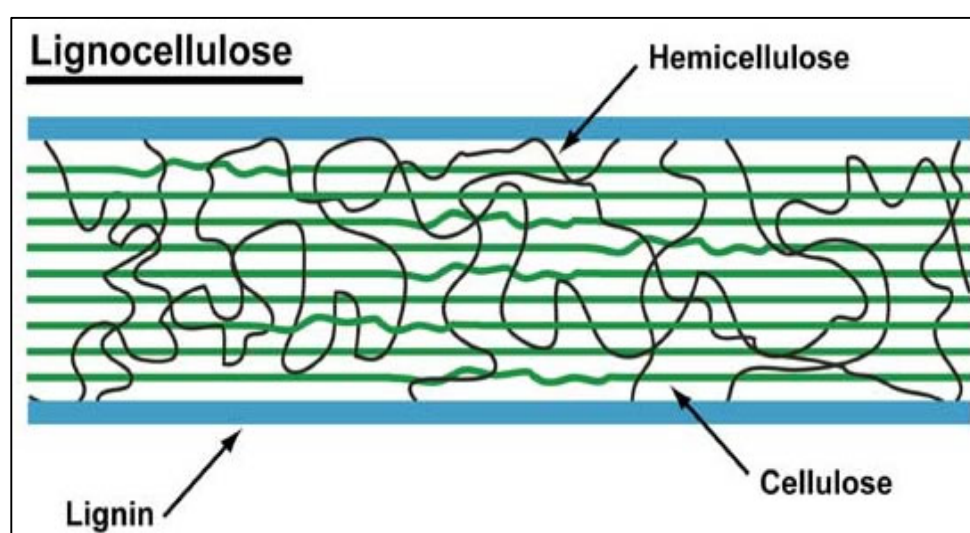


**Figure 1.5:** Different biomass sources which can be used to produce renewable fuels and chemicals.

Biomass is the most commonly utilised raw material in the production of renewable chemicals and it has several advantages over fossil resources for production of fuels and chemicals. They can be replenished within an annual cycle or within a generation, unlike fossil fuels which cannot be created by man's activities and takes millions of years to form. They are also more evenly distributed globally than fossil resources. Most importantly, renewable raw materials do not result in a net increase in GHG emissions, and so decrease environmental pollution and degradation (Gupta and Demirbas, 2010).

### 1.2.6.1 Lignocellulose

Lignocellulose is the term used for biomass from woody or fibrous plant materials (Sims *et al.*, 2008), being a combination of lignin, cellulose and hemicellulose (**Figure 1.6**). Although biomass with high starch content such as cereal bran and sago pith waste are also sometimes described as lignocellulosic biomass, these are more ideally described simply as biomass or specifically as starchy biomass. They encompass primary agricultural wastes such as straw, secondary agricultural wastes such as rice husks, wood and forestry wastes and energy crops among others (**Figure 1.5**).



**Figure 1.6:** Simplified structure of lignocellulose

**Cellulose ( $C_6H_{10}O_5$ )<sub>n</sub>:** Cellulose is the most abundant renewable biological resource on earth (Zhang and Lynd, 2006) and is found in bacteria and the cell wall of plants. Its basic monomeric unit is glucose bound successively to form a long chain of repeating  $\beta$ -(1 $\rightarrow$ 4) bonds, with cellobiose, a dimer of glucose, referred to as the basic chemical unit of cellulose (Varshney and Naithani, 2011). The chains are packed into crystalline microfibrils (**Figure 1.6**) within which the cellulose exists in both amorphous and crystalline forms. The degree of crystallinity in cellulose determines the rate and yield of bioconversion of lignocellulosic materials into sugars, with a decrease in crystallinity being more favourable to the process.

**Hemicellulose ( $C_5H_8O_4$ )<sub>n</sub>:** Also known as polyoses, hemicellulose is a short-chained, branched, heterogeneous polymer of polysaccharides and polyuronides. Hemicellulose

polysaccharide contains different polymers joined in  $\beta$ -(1 $\rightarrow$ 4) linkages of hexosans and polymers of pentosans. The polyuronide groups are more susceptible to chemical and enzymatic treatments than the polysaccharide component of hemicellulose thus making hemicellulose easier to hydrolyse into component sugars than cellulose (Karimi *et al.*, 2013).

**Lignin**  $[(C_9H_{10}O_3)(OCH_3)_{0.9-1.7}]_n$ : is a very complex phenyl propane polymer that contains many functional groups such as hydroxyl, methoxyl and carbonyl. It is believed that during plant biosynthesis, lignin is not simply deposited between cellulose and hemicellulose, but linked, at least partially, with them thus acting as a kind cement or glue (Karimi *et al.*, 2013). Unlike cellulose and hemicellulose, lignin cannot be utilized in most fermentation processes as it can only be degraded by a few organisms (Sims *et al.*, 2008).

Since the term lignocellulosic biomass is used to describe a heterogeneous group of materials, as is evident in **Table 1.1**, its composition depends on its source, with varying proportions of cellulose, hemicellulose, starch and lignin (De Canio *et al.*, 2011; Cardenas-Toro *et al.*, 2014).

There are also a number of other materials found in lignocellulosic materials, the composition and content of which vary between materials, and which can be readily removed using solvents. They are described as extractives such as resins, and non-extractives such as pectin based on their solubility in water (Karimi *et al.*, 2013).

Lignocellulosic biomass is recalcitrant and releasing the sugars from it requires severe pretreatment steps, and is accompanied by the release of compounds inhibitory to subsequent fermentation (Kamm, 2014). The production of renewable biocommodities from lignocellulosic material is thus largely dependent on the ability to make the carbohydrate content available to hydrolysing enzymes or chemicals, and the robustness of the fermenting organism.

**Table 1.1:** Composition of some biomass materials.

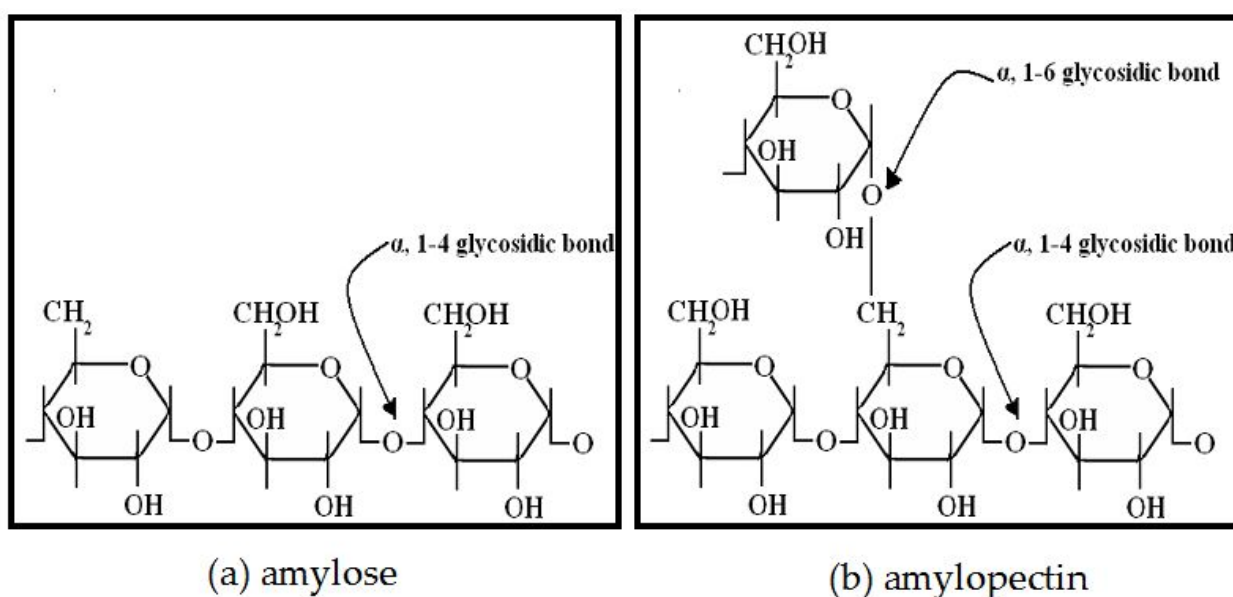
SOURCE	CELLULOSE	HEMICELLULOSE	LIGNIN	STARCH
Poplar	49.0	17.0	18.0	-
Eucalyptus	43.3	31.8	24.7	-
Maize stalk straw	38.0	26.0	11.0	-
Wheat straw	34.0	27.6	18.0	-
Rice straw	37	22.7	13.6	-
Miscanthus straw	44.7	29.6	21.0	-
Cane bagasse	39.7	24.6	25.2	-
Cassava pulp	-	-	-	79.5
Oil palm shells	39.7	21.8	32.5	-
Rice husk	25.4	49.6	22.8	-
Hemp	37.5	27.5	22.0	-
Cassava flour waste	-	-	19.0	60.0
Soybean stalk and leaves	32.1	18.5	-	-
Tomato plant waste	25.7	6.0	19.5	-
Switchgrass	42.9	23.5	21.8	-
Beech wood	45.1	31.5	22.3	-
Rapeseed straw	49.2	14.6	21.6	-
Ginger bagasse	-	-	-	56.5
Agrarian residues	32.0	17.0	-	-

#### 1.2.6.2 Food Wastes - Starch

Among the various sources of biomass (**Figure 2.1**) food waste is of particular importance in producing biocommodities for various reasons. Firstly, because of increasing population, food production and consumption is increasing while due to urbanisation disposal systems are lagging behind thus resulting in indiscriminate disposal of food waste with about 90 million tonnes of food waste being generated in the EU in 2010 alone. Also being nutritionally-rich, food wastes are liable to putrefication and can cause considerable environmental problems (Ravindran and Jaiswal, 2016). Food wastes contain high amounts of cellulose, hemicellulose and lignin (**Section 2.6.1**), but in addition, they also contain starch.

**Starch ( $C_6H_{10}O_5$ )<sub>n</sub>:** Starch is one of nature's most abundant carbohydrates found in root crops, cereals and some fruits as an energy store (Cardenas-Toro *et al.*, 2014) and in the

stems of some trees like the pith of the sago palm. It is the second major component of biomass after cellulose. It is also a polymer of glucose and contains crystalline and amorphous regions. Starch consists of amylose and amylopectin polymer molecules. Amylose is mostly linear and comprises chains of  $\alpha$ -(1 $\rightarrow$ 4) glucopyranose units while amylopectin is considerably branched with the main chains of  $\alpha$ -(1,4)-glucopyranose connected by  $\alpha$ -(1 $\rightarrow$ 6) linkages (**Figure 1.7**).



**Figure 1.7:** The two polymers that make up starch, amylose and amylopectin (Chhabra, 2014).

Acid hydrolysis of the starch biomass fragments these chains resulting in the formation of oligosaccharides and the glucose monomer. Starchy biomass is not as widely used as lignocellulosic materials but is easier to hydrolyse into glucose because of its  $\alpha$ -linkages (Cardenas-Toro *et al.*, 2014).

In addition, like agricultural wastes, food wastes are available in large quantities in the short term. For instance with a production of about 7 m metric tonnes of sorghum in 2015, Nigeria is the world's second largest producer ([www.worldsorghumproduction.com](http://www.worldsorghumproduction.com), 2016), and Africa's largest of this commodity. Almost all of this sorghum is locally processed and consumed but there is no formal value-addition program for the waste generated, so it is

discarded into the environment or fed to animals as part of their feed regime. However the conversion of biomass waste to bulk chemicals was found to be nearly 7.5 and 3.5 times more profitable than its conversion to animal feed or transportation fuel respectively (Tuck *et al.*, 2012). There is thus an economic benefit to enhancing the utilisation of these wastes.

### **1.2.6.3 Sorghum Biomass**

Like maize, sorghum has been exploited for the production of renewable energy and chemicals in several ways. This section gives a brief overview of the sorghum plant and its applications and potential for use as a renewable biomass.

#### **Sorghum**

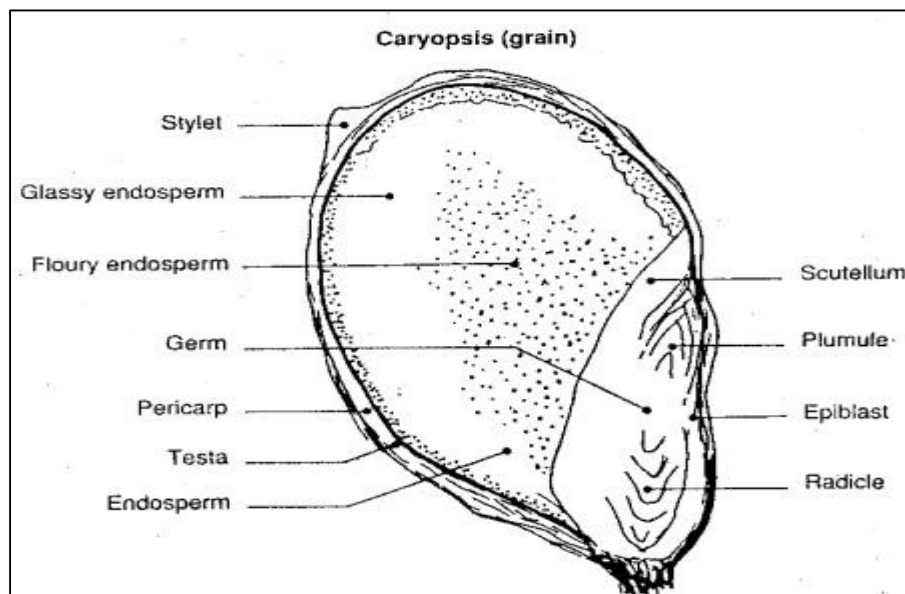
Sorghum (*Sorghum bicolor* (L.) Moench) is a cereal which grows well in harsh environments where other crops grow or yield poorly (**Figure 1.8**). It belongs to the grass family Poaceae and the same tribe Andropogonae as sugar cane and they are thus close relatives. Some sorghum varieties are cultivated primarily for the juice in their stems which is rich in glucose and sucrose, harnessed for 1<sup>st</sup> generation bioprocessing and are called sweet sorghum. However, the most widely cultivated varieties are grain sorghums native to Africa with full grain heads which can contain up to 74 % starch by kernel weight (FAO, 1995).

*Sorghum bicolor* is cultivated as an annual and is divided into five basic races: bicolor, guinea, caudatum, kafir and durra (Harlan and De Wet, 1972), but it displays remarkable genetic variability with over 30,000 varieties present globally (Fuleky, 2009). *Sorghum bicolor* is the fifth most important grain in terms of human foodstuff consumption after rice, wheat, maize and barley, and has been a staple in semi-arid regions of Africa and India for centuries because it has a limited requirement for water and fertilizer inputs (FAO, 1995). In Nigeria it is grown towards the end of the rainy season, from around September, usually after maize has been harvested.



**Figure 1.8:** Mature grain sorghum crops in the field. Source [www.agric.wa.gov.au](http://www.agric.wa.gov.au)

The sorghum kernel is a caryopsis with the pericarp being completely fused with the endosperm (**Figure 1.9**). The endosperm is the largest component of the sorghum kernel and contains the starch granules and protein bodies.



**Figure 1.9:** The sorghum caryopsis (NRI, 1996).

The pericarp usually contains a pigment, and in some genotypes, a testa may be present and may also be pigmented (NRI, 1996). Sorghum kernel colour is due to the presence of phenolic flavanoid pigments, anthocyanins and flavan-4-ols which are located in the pericarp.

### Sorghum Classification

The presence of tannins influences the appearance of sorghum varieties. Sorghum is classified based on tannin content into types I-III. Type I sorghums are those types in which no significant amount of tannins can be extracted with acidified methanol. Type II contains tannins which can only be extracted with acidified methanol but not with pure methanol. Type III sorghum contains tannins that can be extracted with both ordinary methanol and acidified methanol.

Sorghum is also classified based on colour into four types: white, red, brown and black sorghums. White sorghum is referred to as “food type” and contains no detectable amounts of tannins and anthocyanin and low amounts of extractable phenol. This variety is commonly marketed as a replacement for wheat in ethnic foods, cakes biscuits etc. and targets sufferers of celiac disease (an allergy to wheat gluten) or people avoiding gluten in their diets. Red sorghums possess a red pericarp with a significant amount of phenols, but do not contain tannins. Brown sorghum contains large amounts of tannins, a pericarp pigmented over a range of colours and a pigmented testa. Black sorghum contains considerable amounts of tannins and very high levels of anthocyanins (Dunford, 2012).

It is erroneously believed that all sorghums with a red/brown pericarp contain tannins. Sorghums with white, yellow, red, or brown colour pericarp may or may not have tannins depending upon the presence of a pigmented testa, thus pericarp color and its intensity is not a good indicator of tannin content (Dykes and Rooney, 2006). However, the red to moderately dark-coloured sorghums are preferred in several parts of Nigeria for native dishes like *tuwo* and *ogi* because of their unique pink-red appearance and enhanced flavour. High tannin varieties have higher malting potential than the white varieties (NRI, 1996) and several billion litres of malt are produced in Africa annually (Dunford, 2012).



## Bioactive compounds in Sorghum

Sorghum contains several bioactive compounds notably phenolic acids. All sorghum varieties contain these phenolic acids, several contain flavonoids while most do not contain condensed tannins (Dykes and Rooney, 2006).

There are two classes of phenolic acids: hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids are derivatives of benzoic acid and include gallic, p-hydroxybenzoic, syringic, vanillic, protocatechuic acids etc. The hydroxycinnamic acids include coumaric, caffeic, ferulic, and sinapic acids (Dykes and Rooney, 2006).

Tannins are high molecular weight phenolic compounds found in sorghum grains with a pigmented pericarp or testa. Tannins and anthocyanins are the major phenolic compounds and flavonoids in sorghum respectively and play a role in plant defence against pests, moulds and insects (Dunford, 2012). Condensed tannins, also known as proanthocyanidins or procyanidins, are high-molecular weight polyphenols. They are polymers of flavan-3-ols linked mainly through C4 → C8 bonds; with the presence of a C4 → C6 linkage being known as B-type linkage, and an additional C2 → C7 ether bond resulting in an A-type linkage (Preedy, 2012). Predominantly the B-type proanthocyanidins are found in sorghum while A-types are found in cranberries. The sorghum proanthocyanidins have (-)-epicatechin as extension units and catechin as terminal units. Tannic acid is hydrolysable and has never been isolated from sorghums (Dykes and Rooney, 2006).

Some sorghum bioactive compounds can have valuable food uses and others have considerable health benefits. For instance, its unique anthocyanins can be used as food colourants, its 3-deoxyanthocyanins being stable pigments at high pH values. The tannin-rich sorghums have excellent antioxidant properties comparable to those of fruits and vegetable (Dykes and Rooney, 2006). In addition, they have high dietary fibre and have lower digestibility due to the nature of their amylose and amylopectin (FAO, 1995), thus they can provide a feeling of satiety or fullness. However, condensed tannins can form complexes with proteins in food and with proteases in the alimentary tract thus reducing protein digestibility (Dunford, 2012).

## Sorghum in Renewable Bioprocessing

Several industrial applications of sorghum exist and these are mainly in the food industries where the nutraceutical properties of sorghum are exploited. Grain sorghum is used to produce starch, biopolymer films, edible wax and coatings. Sorghum kafirin is a potential feedstock for biopolymer production and its films have been shown to favourably compare with those made from maize [Da Silva and Taylor, 2005 in (Dunford, 2012)] with widespread interest in this product. Processing sorghum for the extraction of starch in domestic or industrial uses is carried out by wet milling. This process produces starch that is coloured with the pigments from the pericarp, and testa. While this colour is desirable in some native Nigerian foods, Dunford et al. (2006) report that it is undesirable industrially and several techniques such as bleaching and low cost abbreviated milling are employed to remove the stain.

Stalks of the sweet sorghum variety contain several carbohydrates such as glucose and sucrose in the juice, in addition to cellulose and hemicellulose in the bagasse, thus the fermentation of sweet sorghum biomass into bioethanol has a higher maximum theoretical yield than just glucose (0.51 g ethanol /g glucose).

Sorghum whole grains and the bran from the dry-milling method have been employed in ethanol (Corredor *et al.*, 2006; Corredor *et al.*, 2007) while the bran from wet-milling has been used for enzyme production (Abu, 2004) because they contain residual fermentable starch. Tannin removal from sorghum grains has been shown to significantly reduce peak and final viscosities of sorghum grain mashes. However, higher viscosities were shown to result in higher ethanol yields (Zhao *et al.*, 2008). The presence of tannins in sorghum could thus directly contribute to higher ethanol production from sorghum than other grains. There are no reports found of bio-based chemical production from sorghum bran, it is thus a biomass material with as yet untapped potential in this area.

### 1.2.7 Pretreatment of Biomass

The first step in the production of 2<sup>nd</sup> gen bio-products from most biomass types is size-reduction and pretreatment (Kim and Dale, 2004) in order to make the biomass more amenable to hydrolysis (often by enzyme treatment). Pretreatment is defined by Zheng et al. (2009) as a process that converts lignocellulosic biomass from its native form, in which it is recalcitrant to cellulase enzyme systems, into a form for which cellulose hydrolysis is much

more effective. Pretreatment is required to alter the macroscopic and microscopic size and structure of biomass as well as its chemical composition to facilitate rapid and efficient hydrolysis of carbohydrates to fermentable sugars while hydrolysis specifically refers to those processes that convert the carbohydrates into monomeric sugars (Zheng *et al.*, 2009). However, in common parlance ‘pretreatment’ is used to include any treatments performed to biomass to convert it to fermentable sugars, with hydrolysis also widely referred to as a pretreatment.

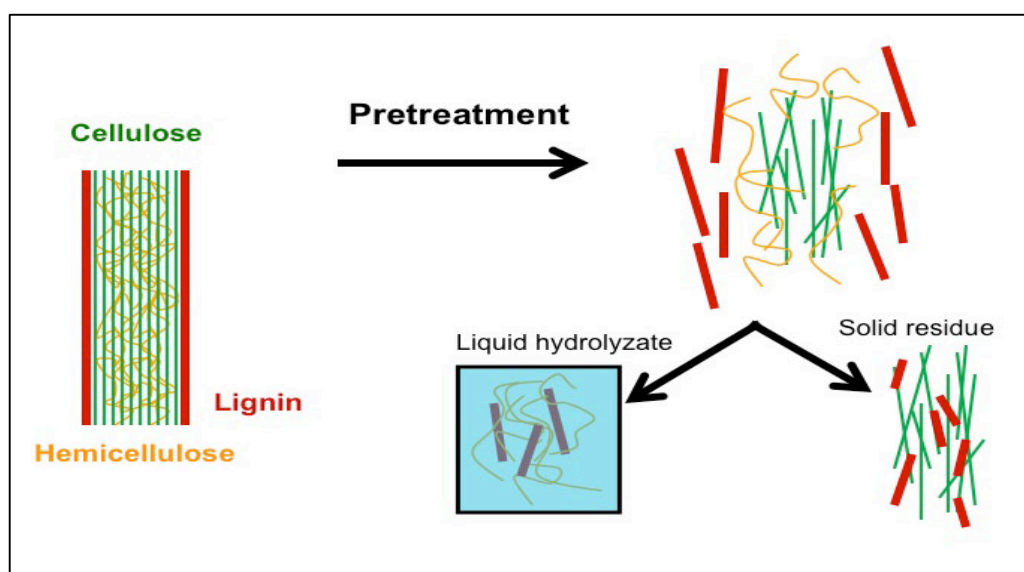
There are generally four main categories of pretreatments viz: physical, biological, chemical and physico-chemical pretreatments. These have been extensively discussed in several reviews (Mosier *et al.*, 2005; Yang and Wyman, 2008; Alvira *et al.*, 2010).

Physical pretreatments do not use any chemical agents and include particle size reduction (e.g. milling), extrusion, steam explosion and liquid hot water treatment. They mostly aim to increase surface area, decrease crystallinity and generally facilitate accessibility of biomass to enzymes. Chemical pretreatments involve the use of chemicals to attack various components of biomass. Acid pretreatment is used to solubilize the hemicellulosic fraction of the biomass thus making the cellulose more accessible to cellulases, and involves the use of mineral acids such as sulphuric, phosphoric, hydrochloric and nitric acids. It can be achieved using dilute or concentrated acids but corrosion and inhibitor formation are major drawbacks of the using the latter. Thus, dilute acid pretreatment is one of the most widely used and studied methods for industrial applications with high saccharification yields reported. Alkali pretreatment employs the use of sodium, potassium, calcium and ammonium hydroxides and increases cellulose digestibility and is widely used for lignin solubilisation. Ionic liquids are salts which exist as liquids at low temperatures and are termed “green solvents” as they leave no toxic residues. They are however expensive and require recovery steps. Biological pretreatment involves the use of microorganisms such as the white-rot fungi *Phanerochaete chrysosporium* to degrade lignin and hemicellulose to increase accessibility of sugars for enzymatic hydrolysis (Alvira *et al.*, 2010).

#### **1.2.7.1 Lignocellulosic Biomass**

The bioconversion of lignocellulosic biomass into sugar monomers is difficult to achieve due to the recalcitrance of lignocellulose in its native form. Pretreatment of lignocellulosic biomass usually refers to the modification steps prior to enzymatic digestion. Most

pretreatment methods thus aim to break up the hydrogen bonds in crystalline cellulose and remove lignin and hemicellulose in order to facilitate subsequent enzymatic conversion (Mosier *et al.*, 2005) (**Figure 1.10**). The hemicellulose is converted to its pentose monomers in solution, the lignin is dissolved while the cellulose remains as a residue which can then be enzymatically hydrolysed into glucose.



**Figure 1.10:** Simple schematic showing how pretreatment breaks lignocellulosic biomass into its components (Clifford, 2015).

However, hydrolysing lignocellulosic biomass into simple sugars is still fraught with challenges because cellulose digestion depends on an interplay of several physical, chemical structural and compositional factors (Alvira *et al.*, 2010).

Due to the considerable heterogeneity of biomass (**Table 1.1**), the unique physico-chemical characteristics of each biomass determine the appropriate pretreatment methods or technologies to employ. There is thus no universal pretreatment regimen as some methods have been shown to be more appropriate for some and not for other feedstocks (Alvira *et al.*, 2010).

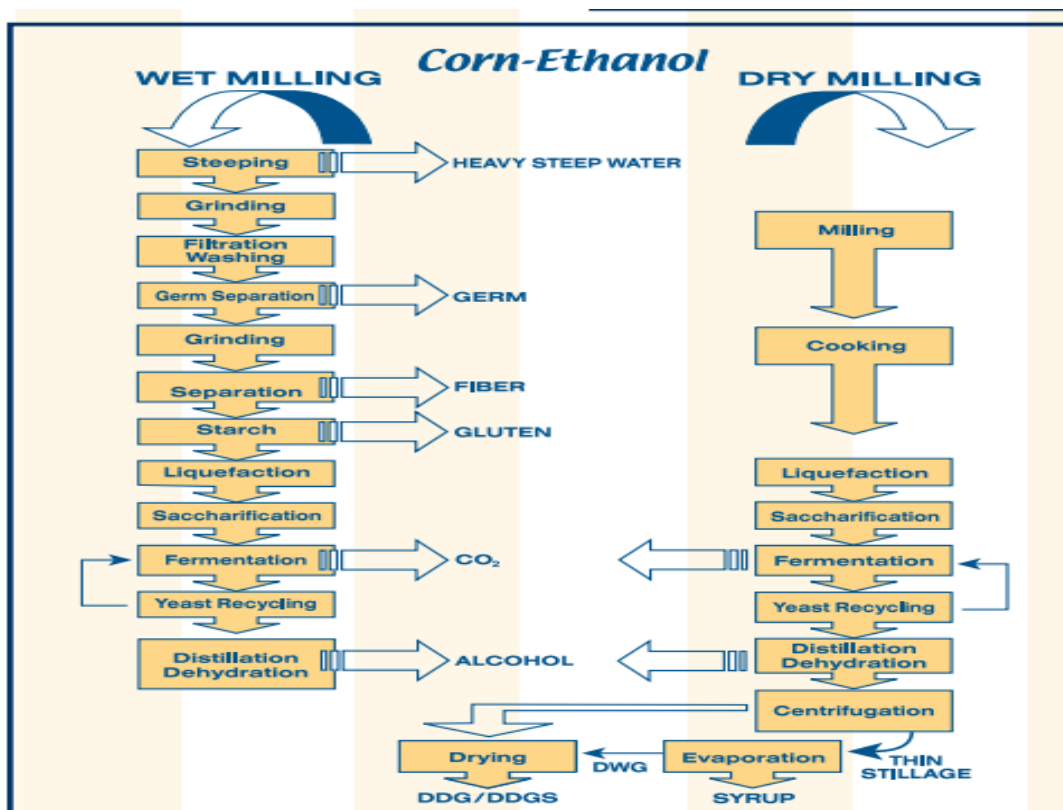
Given its significant impact on overall process economics, several factors must be considered when selecting the pretreatment methods to employ and these include:

- The nature and energy requirements of sample preparation steps such as size reduction required to put the sample in a useable state

- The type of chemicals to be utilised, the need (if any) for subsequent neutralisation, and other processing required prior to fermentation
- The pretreatment process must provide a high concentration of the desired fermentable sugars to keep the process of fermentation and product recovery economical
- The process should neither leave residues that will be toxic to the intended fermenting organism, nor produce fermentation inhibitors from the biomass.

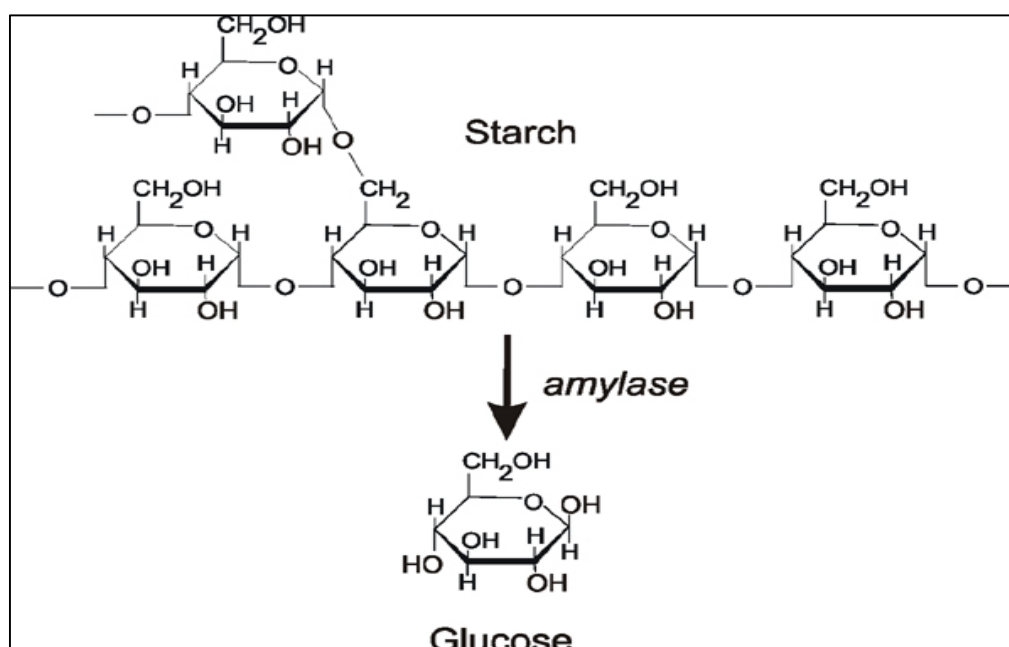
### 1.2.7.2 Starchy Biomass

Conversion of starch-rich biomass to glucose in biorefineries is relatively easier than with lignocellulosic biomass, and it has been well established in the traditional 1<sup>st</sup> gen biofuel industry. There are two methods used to process maize to ethanol in the USA: the wet milling and dry milling methods.



**Figure 1.11:** Wet milling and dry milling processes of ethanol production (AGMRC, 2016)

In the early years of bioethanol production wet-milling was used to produce ethanol from residual starch as a value-addition, after separating desired components such as oil and germ (AGMRC, 2016). Now, dry milling is the most widely used method as it is a quicker process requiring lower capital investment and higher ethanol yields (**Figure 1.11**). In both processes, the starch is subjected to a series of processes that can be described as being analogous to lignocellulosic pretreatment. There is a size reduction process (as mentioned in the previous section) comprising milling/grinding, followed by a cooking process. Starch transitions from its native semi-crystalline state to an amorphous state when cooked in the presence of excess water to a temperature of about 60-70°C; this process, known as gelatinisation, disrupts hydrogen bonds within and between the starch molecules (Warner and Mosier, 2007). Next the gelatinised starch is hydrolysed by amylases at relatively high temperatures in a process known as liquefaction (Balat *et al.*, 2008). The starch is hydrolysed into a mix of glucose (**Figure 1.12**), maltose and dextrins which are low molecular weight carbohydrates. Hydrolysis is then followed by the addition of amyloglucosidase enzyme which saccharifies the dextrins into glucose. The glucose-rich liquid produced from this pretreatment is known as wort, and can then be fermented into ethanol, or other bio-products.



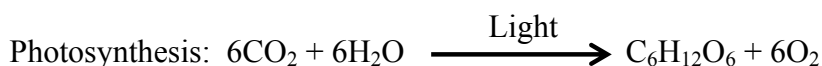
**Figure 1.12:** Enzymatic hydrolysis of starch to glucose ([www.biotek.com](http://www.biotek.com))

### 1.2.8 Renewable Fuels and Chemicals

The use of biomass for energy, materials and chemicals parallels both the concepts of the refinery and sustainability (Lucia *et al.*, 2006). Fuels are the largest group of renewable high-value materials produced while chemical compounds produced from biomass also form an important part of our daily lives and range from simple substances such as water and salt to complex ones such as aspirin, saccharine and detergents.

#### 1.2.8.1 Fuels

**Ethanol** is the oldest synthetic organic chemical used by mankind. It used to be chemically produced solely by the hydration of ethylene (a product of the petroleum refining process) but is now also produced by the fermentation of natural sugars, starches and cellulosic biomass (Gupta and Demirbas, 2010) by *Saccharomyces cerevisiae* and *Zymomonas mobilis* among other organisms. The basic reactions involved in ethanol biosynthesis and combustion are shown below:



Ethanol combusts cleaner than petrol, with fewer particulates.

The manufacturing process adopted (chemical or biological) depends mainly on the prevailing prices of feedstocks, petroleum and grains, and which process is more economical.

**Methane** ( $\text{CH}_4$ ) is a major component of natural gas but is now also produced fermentatively, in combination mainly with carbon dioxide, as biogas by methanogens such as *Methanobrevibacter* sp and *Methanopyrus* sp. It is formed from the anaerobic digestion of sewage sludge, animal wastes, liquid food processing wastes and industrial effluents in the absence of air, over a few weeks. Biogas is an environment-friendly, cheap and safe gas with a variety of applications (Gupta and Demirbas, 2010).

### ***1.2.8.2 Chemicals***

Renewable chemicals are defined in the U.S. congress bill H.R. 4953 as chemicals produced from biomass and used in the production of polymers, plastics, or formulated products excluding food, feed, or fuels (Voegelé, 2012). This definition excludes any chemicals with a bio-based content of less than 25%. These chemicals include monomers, food additives, fuel molecules, amino acids etc.

Some chemicals serve as intermediate building blocks that are subsequently converted into various value-added derivatives, speciality chemicals or other end-products, and are described as platform chemicals. They include sugars, organic acids, fatty acids and syngas among many others. However, in the strict sense platform chemicals are starting blocks or simple intermediates which are central to the production of various high-value intermediates and bio-based chemicals.

A wide range of bio-based platform chemicals are produced from renewable materials ranging from single-carbon molecules such as biogas and syngas, to a mixture of 5-C and 6-C stream of sugars obtained from hemicellulose, to a purely 6-C mixture of sugars obtainable from starch, cellulose and sucrose, and aromatic chemicals derived from lignin and pyrolytic liquids. Erickson et al. (2012) note that many of these chemicals used to be produced from petroleum refining, but are increasingly being produced by fermentative processes. Unlike renewable biofuels which have attracted a great deal of interest among the public, press and policy makers, renewable chemicals have not been so successfully exploited, yet they present a significant opportunity for the increase in sustainable practices at lower capital costs, and with potentially higher returns.

However, since 2004 when the United States' Department of Energy (DoE) published a list of high-value C-3 to C-6 monomers that could be produced as co-products of the biofuel production processes (Werpy et al., 2004), there has been a great deal of interest from the biotechnological industries, researchers and scientists in the production of these chemicals, particularly the final top 12 products, from renewable materials. This has led to the production of several intermediates and end-products of high economic value which find varied applications in industry, as follows.



## Organic acids

Organic acids are a key group of renewable platform chemicals. Many organic acids are accumulated by microorganisms. In fungi, the accumulation of organic acids is due to the incomplete oxidation of a substrate, usually as a consequence of an imbalance of essential nutrients (Kubicek and Karaffa, 2001). Citric acid is the most commercially important organic acid. It is produced by the submerged fermentation of sugars by *Aspergillus niger*. It is a major acidulant, and stabilizer in foods but has applications in cosmetics and chemicals industries (Tsao, 2003; Aftalion, 2010). Another common example is succinic acid, a dicarboxylic acid which exists as a white odourless solid. It is currently manufactured from maleic anhydride but is biochemically produced in a mixed culture fermentation of glucose in which a bacterial culture converts fumarate generated by *Rhizopus sp* into succinate (Tsao, 2003). Itaconic acid is also widely produced from sugars using *Aspergillus terreus* in submerged fermentations. It has wide industrial applications as a monomer where it confers desirable properties on the finished product. A detailed description of the industrial process by which *A. terreus* is used to produce itaconic acid is provided later (see **Section 4.1.2**).

## Plastics

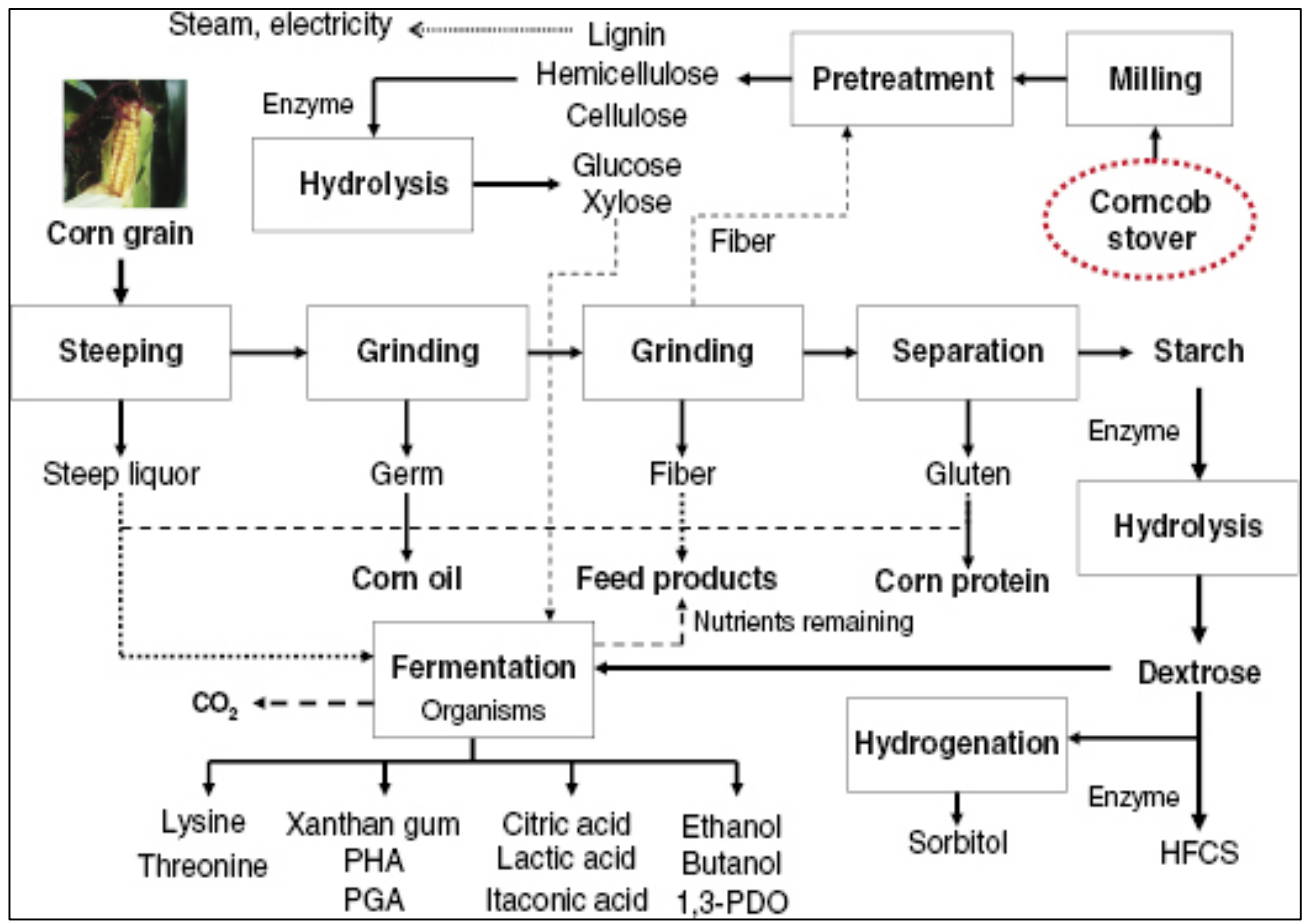
Plastics are also produced using renewable chemicals. For instance bio-derived 1,3-propanediol (PDO) is a key ingredient in the production of over a dozen products. It is fermented from corn sugar and the PDO monomer is separated from the fermentation broth for use in direct product formulations or as an ingredient in polymers. Polylactide from lactic acid is produced by the polymerization of lactide (Demain, 2010), a cyclic di-ester of lactic acid. It is a very commercially successful and versatile source of biodegradable bio-plastics.

The application of novel findings in fermentations and biotechnology have also led to the development of better economies of production and lowered cost. Considering the fact that it was estimated by the World Economic Forum in 2010 that by 2020 the market for biofuels, bio-based bulk chemicals and plastics, and bioprocessing enzymes would approach \$95 billion, the future appears to be very favourable for the biotechnological production of chemicals (King *et al.*, 2010).

### 1.3 Biorefineries

A biorefinery is a term used to describe a facility that integrates biomass conversion processes and equipment to produce fuels, power, heat and high-value chemicals from biomass (Demirbas, 2009). The biorefinery is analogous to a petroleum refinery, producing multiple fuels and products. They produce fuels (low-value product) in high volumes, high-value chemicals in low volumes, while generating electricity and process heat for its own use and perhaps surplus for sale into the power grid. This sustainable processing of biomass into a spectrum of bioenergy and bio-products is vital to achieving a sustainable bio-based economy.

Current biorefineries are predominantly 1<sup>st</sup> gen and utilise traditional sugar- and starch-based whole-crop feedstocks, mainly corn and sugar-cane such as the one shown in **Figure 1.13** (Yang *et al.*, 2013). Most of the existing typical biorefineries can produce one transportation fuel, for example ethanol, from one feedstock such as corn. An integrated biorefinery has the additional advantage of being able to use a wider range of feedstocks and multiple conversion technologies to produce a greater array of products (Dumeignil *et al.*, 2012). Indeed, there is an ongoing transformation of established 1<sup>st</sup> gen biorefineries, as used by traditional agricultural processing companies such as Tate & Lyle and Cargill, into integrated biorefineries with a wide range of fuel and chemical products, often in partnership with oil and chemical companies including Dow Chemical and British Petroleum (Yang *et al.*, 2013). Several other companies such as POET and Abengoa involved in the 1<sup>st</sup> gen programs are beginning to use renewable materials to produce their high value or second generation (2<sup>nd</sup> gen) products (Sims *et al.*, 2008).



**Figure 1.13:** A maize-based biorefinery (Yang *et al.*, 2013).

The high-value chemicals and traditional products enhance profitability, the high-volume fuels help meet national energy needs, while the power production reduces costs and avoids greenhouse-gas emissions.

Europe for instance has commissioned the European Multilevel Integrated Biorefinery Design for Sustainable Biomass Processing or EuroBioRef project, an ambitious project which aims to enable widening biorefinery implementation all across Europe while adapting to local resources and conditions. The project comprises several principles which must be included in the proposed flexible and integrated, zero-waste biorefinery by providing a variety of biomass feedstocks and producing a range of bio-based products in line with the future bio-economy based European society, thus effectively uniting the agriculture and chemical industries (Dumeignil *et al.*, 2012).

## 1.4 Problem Statement

It has become obvious that the climate is being altered by fossil fuel use. Therefore renewable commodities are being investigated to see if it is possible to reduce this impact while also ensuring that the food chain is not hampered by fuel or chemical production. Additionally, the environment is being degraded by petroleum exploration, while food and agricultural processing residues such as sorghum grain waste also litter the environment with no commercial end use. There will thus be a considerable benefit to the climate and the fortune of economies if fossil resources can be replaced with renewable biomass in the production of high-value biocommodities.

## 1.5 Aim and Objectives

The overarching aim of this work is to assess whether sorghum bran, the waste product of sorghum grain processing, can be utilised to produce the value-added chemicals itaconic acid and ethanol, by fermentation. This specific area of research was selected firstly because sorghum bran is a widely available waste material in the sponsor country, Nigeria, of the current research. And secondly because itaconic acid and ethanol are valuable chemicals with likely increasing demand in the future (**Section 1.2.8.2**), whose production by fungal fermentation might be amenable to the use of sorghum bran as a substrate. It was anticipated that such research with itaconic acid and ethanol production might provide a paradigm for the potential of sorghum bran for the production of other high-value chemicals via fermentative processes.

Specific experimental objective of this research were therefore as follows.

- To determine the chemical composition of sorghum bran to ascertain the potential for saccharification and use in fermentation.
- To determine the optimum pretreatment conditions for the production of sugar-rich hydrolysates from sorghum bran for later use in fermentation.
- To determine the types and quantities of any fermentation inhibitors that may be produced or liberated during pretreatment, and assess whether these could be removed.

- To assess whether various species and strains of yeast can use the sugar-rich hydrolysates from sorghum bran as a substrate to produce ethanol during fermentation.
- To assess whether *Aspergillus terreus* can use the sugar-rich hydrolysates from sorghum bran as a substrate to produce itaconic acid during fermentation.
- To attempt to improve the efficiency of fermentation by *Aspergillus terreus* by screening of natural variation in the fungus, together with the application of strain improvement techniques.
- To identify optimum conditions to allow the fermentation by *Aspergillus terreus* of the sugar-rich hydrolysates from sorghum bran to produce itaconic acid.
- To scale up the fermentation and attain stable yields under optimum conditions.

## Chapter 2: General Materials and Methods

The materials and methods employed in more than one chapter of this study are described in this chapter. Materials and methods specific to a particular chapter are described in the relevant chapter where they are applied.

### 2.1 Materials

Sorghum grains were obtained from local markets in Ilorin Nigeria, and processed to obtain bran (**Section 2.2.1**). The red and white varieties (**Section 1.2.6.3**) were used in the preparation of the brans.

All chemicals and media used were reagent grade.

#### 2.1.1 Microorganisms

##### - *Aspergillus terreus* Isolates

Forty six isolates of *Aspergillus terreus* were obtained from the Botany Department University of Nottingham (BDUN) collection of the University of Nottingham. *A. terreus* strain DSM 826 (NRRL 1960, a known high producer of itaconic acid) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and deposited in the BDUN as strain 49-45. A complete list of the strains and their characteristics can be found in **Appendix 1**.

##### - Yeast Species and Strains

Yeast strains (a variety of species – **Section 2.2.3**) employed in the ethanol fermentations were obtained from the University of Nottingham Bioenergy and Brewing Sciences collection. The names and characteristics of the strains employed in this study are shown in **Appendix 2**.

### 2.1.2 Sorghum Bran

The bran (i.e. the hard outer layers of cereal grain, consisting of the combined aleurone and pericarp, and residual starch from the starch extraction process; **Figure 1.9**) was obtained as a waste product of the partially-automated process typically employed in sorghum processing for food production in Nigeria. Two varieties of *Sorghum bicolor* subspecies *bicolor* identified as either the red or white variety by visual inspection of the sorghum kernels were processed to obtain the bran.

### 2.1.3 Defined Ethanol Fermentation Medium

The yeast peptone dextrose (YPD) medium was prepared by dissolving 20 g each of glucose and peptone, and 10 g of yeast extract in 1 L of reverse osmosis water. The pH was adjusted to 5.5 with 5M sulphuric acid and 5M sodium hydroxide and autoclaved at 121°C for 15 minutes. YPD agar was prepared by the addition of 20 g agar before autoclaving.

### 2.1.4 Defined Fungal Fermentation Medium

The defined liquid medium utilised in submerged itaconic acid fermentations was adapted from (Kuenz *et al.*, 2012) and comprised per litre: 120 g glucose, 0.1 g  $\text{KH}_2\text{PO}_4$ , 3 g  $\text{NH}_4\text{NO}_3$ , 1 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.00167 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.008 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.015 g  $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ . Each medium component was prepared separately as a stock solution and the pH adjusted to 3.1 with 2 M  $\text{H}_2\text{SO}_4$ , with the exception of the  $\text{CaCl}_2$  solution which was not pH adjusted. The solutions were sterilised by autoclaving at 121°C for 15 minutes except for the  $\text{FeCl}_3$  solution which was sterile-filtered. The solutions were then mixed to the final concentrations above. Solutions were stored at 4°C pending use.

### 2.1.5 Other Chemical Solutions and Buffers

All solutions and buffers prepared and used throughout this study were prepared in volumetric flasks using deionised water, unless otherwise stated.

#### - Dilute Mineral Acids

1%, 2% and 3% w/w solutions of sulphuric acid were prepared as v/v solutions by making 5.72 ml, 11.44 ml and 17.16 ml respectively of 95 % w/w sulphuric acid up to 1 L; while

nitric acid was prepared similarly as 10.06 ml, 20.12 ml and 30.18 ml of a 70 % solution were made up to 1 L.

Mobile phase for HPLC, 0.005 N sulphuric acid was prepared by dilution of 1.0 ml of 5 M sulphuric acid up to 2 L using reverse osmosis water.

#### **- 10 M Sodium Hydroxide solution**

Four hundred grams of sodium hydroxide pellets was made up to one litre with water in a volumetric flask.

#### **-Standard Solutions**

A 50 g/L stock solution containing glucose, arabinose, galactose and xylose was prepared by dissolving 50 g each of these sugars in 800 ml of water in a 1L volumetric flask. The stock solution was mixed thoroughly to ensure complete dissolution and then made up to a final volume of one litre with deionized water. This solution was then diluted into 100 ml portions of different concentrations as shown in **Table 2.1** below. A standard solution of itaconic acid was also similarly prepared and diluted.

**Table 2.1:** Preparation of 1L of standard solutions of simple sugars and itaconic acid

Concentration	Stock solution	DI Water
(g/L)	(ml)	(ml)
50	100	0
40	80	20
25	50	50
15	30	70
10	20	80
5	10	90
2.5	5	95



All solutions were sterile-filtered through Whatman GD/X syringe filters with 0.2  $\mu\text{m}$  pore size (Whatman International Ltd., UK), dispensed into HPLC vials and stored at  $-20^{\circ}\text{C}$  pending use.

## 2.2 Methods

All commercially-obtained media including Potato Dextrose Agar (PDA) were prepared according to manufacturers' instructions. Media were sterilized at  $121^{\circ}\text{C}$  and 15 psi for 15 min unless otherwise specified.

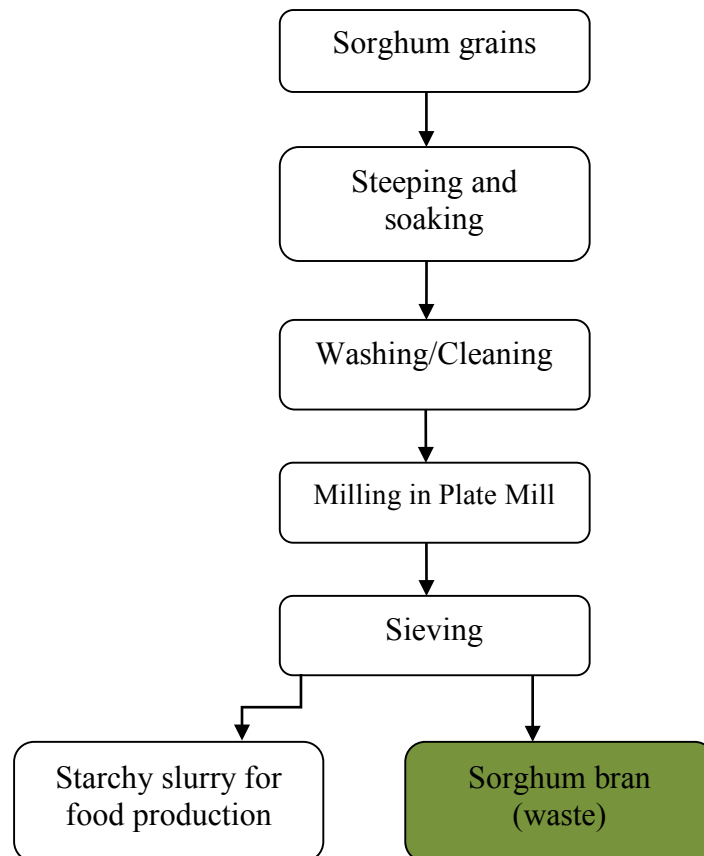
### 2.2.1 Wet-Milling of Sorghum Grains

Sorghum bran used for these experiments was prepared in the typical way it is produced in Nigeria. The grains were washed and then steeped in four times volume of water for 2 days at room temperature. They were then washed again and wet-milled in a conventional petrol-powered wet-mill until a smooth slurry was obtained (**Figure 2.1**).



**Figure 2.1:** A typical petrol-operated Burr-plate mill for wet-milling sorghum for wet-milling of grains and pulses in Nigeria.

The slurry is sieved through muslin cloth to remove the starchy endosperm material, leaving the bran residue which was then sun-dried. The process is illustrated in the flowchart below (**Figure 2.2**):



**Figure 2.2:** Typical process of sorghum bran preparation from the wet-milling method  
The sun-dried bran is stored in sealed polythene bags at room temperature pending use.

### 2.2.2 Storage of Microorganisms

*Aspergillus terreus* isolates were maintained on PDA slants at room temperature for short term storage while the yeast species were maintained on YMA slants at 4°C. All strains were subcultured regularly to maintain viability.

Stocks of all isolates obtained from outside the BDUN collection were grown up and deposited in the collection as reference stocks. For long term storage, a spore/cell suspension of each strain was prepared aseptically and transferred into a 2 ml cryovial. For *A. terreus*, an equal volume of sterile 50 % glycerol solution was added to the suspension, mixed by inversion and capped. These were then labelled and stored in liquid nitrogen vats. The yeasts were stored as stocks in 50 % glycerol solution at -80°C pending use.

### 2.2.3 Preparation of Microorganism Cultures on Solid Media

#### - *Aspergillus terreus* isolates

A sterile swab was used to aseptically transfer *A. terreus* mycelia and/or spores from a pre-existing culture or liquid nitrogen stocks to PDA in 9 cm Petri dishes. They were incubated for five days at 37°C after which the resulting colonies were subcultured onto PDA slants in 30 ml Universal containers, loosely capped and incubated for a week. Dishes were sealed with Parafilm M<sup>®</sup> (Bemis Inc, Wisconsin USA) a plastic paraffin film (where needed), and slant caps tightened and stored at room temperature.

#### - Yeasts

Strains of *Saccharomyces cerevisiae* NCYC 2592, *Scheffersomyces stipitis*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus*, *S. cerevisiae* NCYC 1383, *S. pastorianus*, *S. cerevisiae* NCYC 1119 and *Candida arabinofementas* NCYC 2916 were investigated for their abilities to grow on and ferment sorghum bran hydrolysates into ethanol.

Stock cultures of the yeasts stored in -80°C were revived by streaking on YPD agar plates and incubated at 30°C for 48 hours to obtain actively growing cells. Representative yeast colonies were then used to inoculate 10 ml of YPD broth and incubated at 30°C and 150 rpm for 48 hours. The cells were harvested by centrifugation at 4,000 rpm for 5 min and the

supernatant was discarded, the pellets washed with sterile reverse osmosis water and centrifuged at 17,000 x g at 4°C. The washing was performed three times to remove any traces of the medium before re-suspending the cells in 5 ml of sterile distilled water. They were used directly as the base suspension of cells used for screening tests and as inocula for fermentations (Greetham *et al.*, 2014).

#### **2.2.4 Itaconic Acid Fermentation**

Submerged itaconic acid fermentation was carried out using shake flasks in all experiments unless otherwise stated, and the process is described below.

##### **2.2.4.1 Spore Suspension Preparation**

In a Class II Biosafety cabinet 0.05% Tween 80 solution was added to a recently prepared mature slant of *A. terreus* and the surface of the growth scraped gently with a sterile cotton swab, with care taken to avoid dislodging mycelial bits or clumps. The cinnamon-coloured suspension was then aseptically transferred into a 5 ml universal tube. The suspension was diluted appropriately with 0.05% Tween 80 solution, with the concentration of conidiospores determined microscopically using an improved Neubauer hemocytometer.

##### **2.2.4.2 Shake Flask Fermentation**

Shake flask fermentations were performed in sterile 250 ml Erlenmeyer flasks into which exactly 25 ml of sterile media i.e. the defined media (**Section 3.1.4**) or the sorghum bran hydrolysates (**Section 4.3.2**) were dispensed in the biosafety cabinet. The concentrated spore suspension was used to inoculate the media to a final concentration of  $1 \times 10^6$  spores/ml. The flasks were sealed with sterile polystyrene plugs and then transferred to either a Gallenkamp 10X 400 incubator (Sanyo Gallenkamp Plc; UK), a New Brunswick<sup>TM</sup> Innova® 44 incubator (Eppendorf AG USA) or to a New Brunswick<sup>TM</sup> G25 incubator (New Brunswick<sup>TM</sup> Scientific Co). The incubators were pre-set to a specified temperature and agitation rate for varying periods of time.

For time point experiments, samples were collected aseptically at regular intervals. Flasks were collected from the incubator one at a time, agitated thoroughly to minimise the likelihood of oxygen deprivation in the ongoing fermentation, sampled aseptically, and returned with minimal delay to the incubator.

Samples were then syringe-filtered and evaluated by high performance liquid chromatography (HPLC) as described in **Section 2.2.6**.

#### **2.2.4.3 Mycelial Mass Determination**

The amount of fungal biomass produced was determined at regular intervals during the *A. terreus* fermentations, or upon conclusion was determined as described previously (Kim *et al.*, 2002). Briefly, the fermentation broth was centrifuged and the liquid kept for analysis. The mycelia was then rinsed with sterile distilled water to dissolve any residual sugars and fermentation products, centrifuged at 10,000 rpm and decanted, then transferred to a aluminium foil tray and dried in a convection oven at 70°C till a constant weight was attained. The observed mycelial mass was then recorded.

#### **2.2.5 Ethanol Fermentation**

The pH of all the hydrolysates were adjusted to 5.5 and sterile filtered with a Stericup® 0.20 µm filter. Next, 25 ml was aseptically transferred to pre-sterilised Wheaton® glass bottles containing magnetic stirrers and the yeast cells (prepared as in **Section 2.2.3**) were added at a rate of  $1 \times 10^7$  cells/ml. The bottles were then capped with sterile butyl plugs and covered with crimp-top aluminium caps with a 5.5 mm hole. The metal caps were held in place with a hand-held vial crimper to ensure anaerobic conditions. Sterile Bunsen valves attached at one end to a hypodermic needle was pushed through the butyl plug to enable the release of the carbon dioxide produced in the sealed system, through the slit in the rubber tubing. The mini fermentation vessels (mini-FVs) were then placed on a magnetic stirrer plates set at 200 rpm per minute and incubated at 30°C.

The initial weight was noted and the weight loss from carbon dioxide formation recorded at regular intervals until constant weight. Once there was no more observed weight loss, the experiment was terminated.

For the time point experiments, the number of mini-FVs set up was equal to thrice the number of desired time points. So, for 10 time points, an experiment of 30 mini-FVs were set up under the exact same conditions. At every time point, 3 vessels were terminated by weighing them, opening them and taking out samples aseptically for cell counts and HPLC analyses.

### 2.2.6 HPLC Analyses

Prior to HPLC analyses, all standards and samples were filtered with 0.2 µm Minisart® membrane filters. The fermentation samples were first centrifuged to separate the broth from the mycelia (**Section 2.2.5**) then the broths were syringe-filtered into the HPLC vials. With highly concentrated fermentation broths such as at the early stages of the fermentation when the glucose content was still high, samples were appropriately diluted prior to HPLC analyses.

A modular High Performance Liquid Chromatography (HPLC) setup was used to determine the sugar content of sorghum hydrolysates and itaconic acid yields of fermentation broths. The HPLC system comprised an AS-2055 auto-sampler, PU-1580 pump and RI 2031 refractive index detector (Jasco International Co. Essex, UK). The sample injection volume was 10 µL and the analytes were separated using a Hi-Plex H<sup>+</sup> column (7.7 mm x 300mm; Agilent Technologies, Inc., USA) heated to 45°C in a Hewlett Packard 5890 Series II GC oven (Hewlett Packard, CA, USA). The mobile phase of 0.05 N H<sub>2</sub>SO<sub>4</sub> solution in HPLC grade water was set to flow at a rate of 0.4 ml/min with air bubbles removed using a Jasco degasser. Sample analysis was completed within 25 min and the data was captured and integrated using version 4.6.0.0 of the Azur software package (DATALYS, Saint-Martin-d'Hères, France). Data obtained was processed with the Excel 2010 software (Microsoft Corp., Redmond, WA, USA) where regression equations from standard curves prepared for the standard solutions were used to estimate the concentration of the analytes in the sample.

### 2.2.7 Statistical Analyses

Experiments were performed in triplicate unless otherwise stated, and data is presented as averages with the standard deviation. Charts were plotted with Microsoft® Excel software and statistical analyses performed with the Statgraphics® Centurion XVI software (Statpoint Technologies, Inc., USA).

## Chapter 3: Sorghum Bran Characterisation and Pretreatment

### 3.1 Introduction

In this chapter, sorghum bran prepared as described in **Section 2.2.1** was investigated to determine its chemical characteristics and suitability as a feedstock. Various pretreatment methods were then used to try and produce sugar-rich hydrolysates.

#### 3.1.1 Sorghum Bran Composition

Sorghum is commonly processed for *ogi*, *koko* and *kunu* by wet milling in Nigeria (**Section 2.2.1**). The starchy endosperm is disrupted in this process and the starch solution filtered out from the slurry. Sorghum bran usually bears the colour of the grains from which they were derived, thus bran from red grains will appear pinkish-red and bran from white grains will appear white. However, depending on the efficiency of the milling and sieving processes a considerable proportion of the starch may be left unrecovered from the slurry and discarded with the bran. The composition of bran thus varies considerably between batches due to the immense diversity of sorghum varieties, and because of the variability inherent in the procedures of the small-scale at which wet-milling occurs.

Accurate compositional analysis gives an indication of suitability of a biomass as a potential feedstock for a given bioconversion process. The key component of most biomass compositional analyses intended for use in microbial fermentations is the carbohydrate content because this directly correlates with how much of the bioproduct will be produced in the process (Sluiter et al., 2010). Medium requirements in most fermentation include a carbon source which provides both energy and carbon units for biosynthesis, and sources of nitrogen, sulphur and phosphorus. Trace elements are also required while some microorganisms require the presence of vitamins such as biotin in the medium (Waites *et al.*, 2009).

The composition of the sorghum bran was investigated in order to determine its suitability as a feedstock and the residual starch is assayed in the current research as a potential key substrate for renewable bio-production applications. Other important components include protein content and ash content, the latter of which refers to the minerals and elements in the material and moisture content that can affect shelf life and affect the determination of other components (Sluiter et al., 2010). There have been only a few previous studies reporting the

nutritional composition of sorghum bran (Lochte-Watson *et al.*, 2000; Corredor *et al.*, 2007). However, no comprehensive data regarding the composition of sorghum bran obtained from the wet-milling process could be found, and therefore this particular material was investigated in the present study given its widespread prevalence in Nigeria.

### **3.1.2 Sorghum Bran Pretreatment**

Due to the considerable diversity in the nature of biomasses, it becomes necessary to adopt feedstock-specific pretreatment technologies based on a particular material's properties. In addition, given that pretreatment has a considerable impact on the subsequent steps in a bioconversion in terms of types and quantities of inhibitors produced, efficiency of enzymatic and microbial conversions, no one pretreatment method is ideal for all types of biomass (Alvira *et al.*, 2010). Pretreatment strategies for sorghum bran from dry-milling of sorghum have previously been reported in the scientific literature, notably an approach which used various combinations of hot water, starch degradation and enzymatic degradation (Corredor *et al.*, 2007). The cost of biomass pretreatment has been reported to be second only to the feedstock cost in the production of bioethanol production from lignocellulosic sources by the enzymatic route (Mosier *et al.*, 2005), comprising about 18 % to the total cost while also influencing the types and hence cost of downstream processes (Yang and Wyman, 2008). This present work therefore attempts to avoid the utilisation of very expensive chemicals/processes where possible, to improve the economics of the process.

In 1811, Russian chemist G.S.C. Kirchoff discovered that acid hydrolysis of starch produced a sweet syrup and within 20 years, a 115-litre per day syrup plant was built in America (Hobbs, 2009). Dilute acid hydrolysis is now routinely carried out on lignocellulosic materials to dissolve the hemicellulose and lignin fractions into solution and the process normally followed by enzymatic digestion of the residual cellulose. The  $\alpha$ -1,4 linkages in starch are more rapidly hydrolysed than the  $\alpha$ -1,4 linkages in cellulose to release glucose monomers (Reed, 2012). As a result it was decided that dilute acid pretreatment without a subsequent enzymatic hydrolysis process would be investigated for the ability to give a glucose-rich solution suitable for bioconversions as one alternative in the present study. Thus, in the context of this work, the term 'pretreatment' is used to refer specifically to the digestion of the starchy component of the sorghum bran substrate into glucose.



The product of a hydrolysis process is referred to as a hydrolysate. Hydrolysates are produced from biomass following some form of pretreatment as necessary. Starch is readily converted to D-glucose by acid hydrolysis and can attain up to 100% conversion efficiency in dilute solutions, while at higher starch concentrations, a lower yield is obtained due to reversion reactions (Reed, 2012).

Despite its advantages in terms of simplicity and efficiency, acid hydrolysis of starch is declining in popularity because it requires the use of corrosion resistant materials to contain the reaction and potentially expensive energy input for heating. Instead, it is now being replaced by enzymatic processes in the industrial production of corn syrup. The enzymatic hydrolysis usually involves liquefaction with defined amylases, followed by saccharification by amyloglucosidases (**Section 2.6.4.2**). However, some crude fungal amylases have also been reported to convert starch granules directly to glucose (Textor *et al.*, 1998). Enzyme hydrolysis is quite specific and thus avoids the problems of side reactions, as well as not requiring acid resistant containers or great energy input. Therefore, enzyme hydrolysis was also evaluated as an alternative for hydrolysate production in the present study.

### **3.1.3 Fermentation Inhibitors**

Inhibitors are intrinsic compounds or degradation by-products that are produced or released during chemical (e.g. dilute acid) pretreatments and enzyme hydrolysis with the potential to negatively impact fermentation performance (Jönsson *et al.*, 1998). These compounds are usually reported as inhibitors of the ethanol fermentation process which has been the most widely investigated such process.

The main classes of ethanol fermentation inhibitors encountered in biomass conversion are sugar degradation products, namely furfural and 5-hydroxymethylfurfural (HMF) (furans); weak acids; and phenolic compounds. Furfural and HMF are produced from the degradation of pentoses and hexoses respectively under severe pretreatment conditions, and high concentrations of these chemicals can hinder subsequent cell replication (Pandey, et al., 2014). Weak acids e.g. levulinic and formic acids are formed by the de-acetylation of hemicellulose in the biomass, or from sugar and lignin degradation during pretreatment and both result in a significant drop in intracellular pH (Pandey, et al., 2014). The production of this class of inhibitors is difficult to prevent as they are intrinsic to the biomass itself, affecting survival of the fermenting organism. Phenolic compounds, such as vanillin and

syringaldehyde are a major constituent of lignin. They inhibit fermentation by interacting with the cell membrane of organisms, causing a loss of integrity (Pandey *et al.*, 2014). Lower-molecular-weight phenolic acids might behave similar to weak acids and also disrupt intracellular pH by the production of free radicals. In some studies they have been found to be more toxic than furans (Pandey *et al.*, 2014).

These inhibitors can be avoided to a large extent by optimizing pretreatment conditions for each feedstock.

### **3.1.4 Aims**

This chapter describes work to determine the composition of the sorghum bran used in later experiments. It also includes investigation of pretreatment strategies aimed at increasing the yield of glucose-rich hydrolysates for subsequent fermentation; determination of any inhibitors present in sorghum bran or generated during the hydrolysate-preparation process; and to attempt to detoxify the hydrolysates to improve yields.

The specific aims were to:

- Conduct a summative mass closure and in depth compositional analysis of the feedstock sorghum bran, and determine its suitability for bioconversion processes.
- Investigate and compare different pretreatment processes in order to determine the most suitable for the feedstock and bioprocesses.
- Optimise pretreatment conditions to maximise sugar yields in hydrolysates using relatively inexpensive methods while taking into account the production of fermentation inhibitors.
- Determine inhibitors inherent in sorghum bran or produced during pretreatment and assess whether these might be ameliorated.

## **3.2 Materials and Methods**

General materials have been enumerated in **Chapter 3**, only materials and methods specifically utilised only in this chapter are included in this section.

### **3.2.1 Materials**

All chemicals used were of analytical grade.

#### ***3.2.1.1 Preparation of Chemical Solutions***

All chemical solutions were prepared with reverse osmosis water unless stated otherwise.

##### **- Acetyl Bromide solution in Acetic acid (25 % v/v)**

In a fume-cupboard, 25 ml of neat acetyl bromide was measured into a bottle and made up to 100 ml with glacial acetic acid.

##### **- 0.5 M Hydroxylamine-HCl**

Exactly 6.95 g of Hydroxylamine-HCl was measured into a flask and made up to 200 ml with water.

##### **- 0.3 M Sodium Hydroxide solution**

This was prepared thus: 7.5 ml of a 10M sodium hydroxide (NaOH) stock solution was added to 200 ml of water. The solution was then made up to 250 ml.

##### **- Aqueous Dioxane solution (80% v/v)**

Eighty millilitres of pure dioxane was made up to 100 ml with water in a fume cupboard.

##### **-Dinitrosalicylic acid (DNS) reagent**

The DNS reagent was prepared by dissolving 1.0 g of 3,5-Dinitrosalicylic acid (DNS), 200 mg of crystalline phenol and 50 mg of sodium sulphite in 90 ml of 1 % NaOH and then making it up to 100 mL with the 1 % NaOH (Linskens and Jackson, 1999). The reagent was prepared fresh before use.

##### **- 1.2 M Sodium acetate buffer, pH 3.8**

This was prepared by mixing 69.6 ml of glacial acetic acid with 800 ml of water, adjusting the pH to 3.8 using 4M NaOH and then making it up to a final volume of 1 L (Serna-Saldivar, 2012). The buffer was stored at 4°C pending use.

##### **- Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM)**

This was prepared according to instructions from the Megazyme starch test kit manual thus: 5.8 ml of glacial acetic acid (1.05 g/ml) was added to 900 ml of distilled water. The pH was adjusted to 5.0 by the addition of 1 M sodium hydroxide solution. Next, 0.74 g of calcium chloride dihydrate was added and dissolved. The volume was adjusted to 1 L and stored at 4°C pending use.

**- 40% (w/v) Rochelle salt solution**

This was prepared by dissolving 40 g of Rochelle salt (potassium sodium tartrate) in 80 ml of reverse osmosis water and making it up to a final volume of 100 ml using reverse osmosis water (Linskens and Jackson, 1999).

**- 12 N Sulphuric acid**

Concentrated sulphuric acid (96 %) equivalent to 36 N was diluted three-fold with water to obtain a 12 N solution.

**- 72 % Sulphuric acid**

Into a volumetric flask containing 20 ml of cold reverse osmosis water, 75 ml of ice cold concentrated sulphuric acid (96 %) was carefully added and made up to 100 ml with water in a fume cupboard.

**- 80 % Ethanol**

This was prepared by diluting 421.05 ml of 95 % ethanol with reverse osmosis water up to a final volume of 500 ml.

**- 2 M Potassium hydroxide**

This was prepared by dissolving 56.0 g of potassium hydroxide (KOH) in water with the final volume made up to 500 ml.

**- 1 % SDS and 5 % TEA SOLUTION**

A solution of sodium dodecyl sulphate-triethanolamine (SDS-TEA) was prepared by transferring 1g of SDS and 5 % (v/v) of TEA into reverse osmosis water and made up to 100 ml with water.

**- 0.01N Hydrochloric acid solution**

Into a volumetric flask, 1 ml of 5 M hydrochloric acid (HCl) was made up to 100 ml with water.

**- 0.01 M Ferric chloride solution in 0.01 N HCl**

Exactly 1.62 g of ferric chloride ( $\text{FeCl}_3$ ) was dissolved in the 0.01 N HCl solution up to 100 ml.

**- 0.2 M Acetate buffer with 0.17 M sodium chloride**

Into a volumetric flask containing 80 ml of water, 1.143 ml of glacial acetic acid was transferred, and 0.994 g of sodium chloride added. The solution was mixed thoroughly and the pH was adjusted to 5.0 with NaOH solution and made up to 100 ml with water.

**- 1 mg/ml standard protein solution**

A standard protein solution was prepared by dissolving 1mg/ml of serum bovine albumin (Sigma Aldrich, UK) in the solution of 0.20 M acetate buffer with 0.17 M sodium chloride and the pH was retained at 5.0.

**- 0.05M Sodium hydroxide solution**

This solution was prepared by dissolving 2 g of NaOH pellets in water and made up to 1 L.

### **3.2.2 Methods**

The composition of the brans were first determined according to the summative mass closure method and then some specific sub-components elucidated. Next the pretreatment methods for the production of hydrolysates were evaluated and possible presence of inhibitors investigated.

#### ***3.2.2.1 Sorghum Bran Compositional Analyses***

Summative mass closure involves the use of a suite of tests to determine the composition of a biomass material in a way that accounts for 100 % of the material by weight (Sluiter et al., 2010). In order to determine the composition of sorghum brans by a summative mass closure, the review by Sluiter et al. (2010) was used to draft a tailored suite of procedures mainly from the Laboratory Analytical Procedures (LAPs) produced by the National Renewable

Energy Laboratory, USA (NREL). This suite was expected to give an approximately 100% summative mass closure. Where the NREL LAPs could not be followed, other well-established protocols for the determination of that component were implemented instead. In all of the following analyses just one source of WB or RB bran (prepared as **Section 2.2.1**) was analysed. These samples were obtained from Ilorin Nigeria, and used throughout the chapter for consistency.

- Moisture Content (MC)

Prior to other compositional analyses and subsequent processes the moisture contents of each bran was determined in triplicate using a Mettler-Toledo® (HR83) halogen moisture content analyser (**Figure 3.1**).



**Figure 3.1:** The Mettler-Toledo® (HR83) halogen moisture content analyser.

The halogen heating elements were warmed up for approximately 5 min, then an aluminium weighing dish was placed on the balance pan, and the balance tared. A small amount was transferred to the weighing dish, spread evenly and analysed according to the instructions in the operation manual following the “Barley Rap” program. Once the program ended, the percent moisture was recorded. Samples were analysed in triplicate and the analyser left to cool to at least 50°C in between samples.

The subsequent analyses were then determined on ODW bases, unless it is stated that the MC values of samples were determined just before analyses for accuracy. However, 20 g of each

bran was stored as is without oven drying, for analyses that can only be performed with samples that are either air-dried or dried at less than 40 °C.

#### - Sample Preparation

The brans were comminuted in a planetary Ball Mill (Fritsch, GmbH) to obtain a free-flowing powder. Twelve ceramic balls were added to each of two zirconium oxide pots and then filled with bran to about  $\frac{3}{4}$  capacity. The pots were closed and spun at 250 rpm for three cycles of 3 min and two pauses of 2 min (to prevent over-heating).

The milled brans were then sieved through 20-mesh and 80-mesh sieves (Endecotts Limited, UK) on a vibratory sieve shaker and the -20, -80 and -20/+80 fractions weighed. Fractions were stored in cool dry conditions pending use.

#### - Total Ash Content

The total ash content was determined based on an adaptation of the LAP determination of biomass of ash methodology (Sluiter *et al.*, 2005). Briefly, clean porcelain crucibles were appropriately labelled then placed in an oven at 520°C for five h to remove any residual moisture. They were then transferred into a desiccator for one hour to prevent them from absorbing moisture after which their weights were measured and recorded. Next the bran samples were evaluated for moisture content as described in **Section 4.2.1.1.1** and 1.5 g  $\pm$  0.3 mg weighed in triplicates into tared crucibles. The crucibles were then placed in the furnace at 520 °C for 28 hr. The crucibles were carefully transferred to a desiccator while avoiding drafts which could cause mechanical loss of sample, left to cool for one hour then weighed.

The ash content was then determined using the formula below:

$$\text{Total Ash \%} = \frac{\text{Weight of crucible and ash} - \text{Weight of Crucible}}{\text{ODW of sample}} \times 100 \dots \text{Equation 4.1}$$

#### - Carbohydrate Content

Carbohydrates can be structural or non-structural, and they make up a major portion of biomass samples. The structural carbohydrates are bound in the biomass matrix while the non-structural carbohydrates are easily removed by extraction steps. The structural carbohydrates in sorghum bran were determined by a two-step concentrated acid hydrolysis method to fractionate the biomass into easily quantifiable forms. The lignin fractionates into soluble and insoluble lignin, while the carbohydrates are hydrolysed into its monomeric forms which are soluble in the liquid and can easily be quantified (Sluiter *et al.*, 2008). The carbohydrate content was determined according to the method described by Sluiter *et al.* (2008).

Bran samples used for this procedure were oven dried at 30 °C to get the MC below 10 %. In triplicates, exactly 300 mg of each bran was weighed into a tared pressure tube and 3.0 ml of 72 % H<sub>2</sub>SO<sub>4</sub> was added. The mixture was stirred for one min with a Teflon stir rod to ensure thorough mixing. The tubes were then placed in a water bath at 30 ± 1 °C for 60 min with constant stirring every 10 min to ensure uniform hydrolysis. Next the tubes were removed from the bath, and the acid diluted to a 4 % concentration by the addition of 84 ml of deionised water and the tubes capped tightly. The samples were mixed by inverting the tubes severally to eliminate phase separation in acid concentration.

At this point a set of sugar recovery standards (SRS) of glucose, xylose, mannose, maltose, cellobiose and arabinose were prepared by weighing out 0.5 g, 0.1 g, 0.02 g, 0.1 g, 0.02 g and 0.02 g respectively into pressure tubes. Next, 10.0 ml of water was added and tubes shaken to mix and finally 348 µl of 72 % H<sub>2</sub>SO<sub>4</sub> was added. The tubes were capped tightly, placed in same rack as the sample tubes and autoclaved at 121 °C for 60 min at a pressure of 15 psi in order to complete the reaction. Upon completion of the cycle, the hydrolysates were left to cool and the caps removed and an aliquot of 50 ml was transferred into a storage bottle.

The hydrolysates were neutralised with calcium carbonate to pH 5-6 and the pH was monitored with pH paper to ensure it did not exceed pH 6 in order to minimise sugar degradation. Samples were left to settle, then the clear liquid filtered into HPLC autosampler vials and labelled. A set of standards were prepared for a five point calibration standard containing 0.5, 1.0, 2.0, 4.0, 6.0 mg/ml for each of the same sugars above. A calibration verification standard (CVS) was also prepared at 2.5 mg/ml using sugars from a different manufacturer to validate the calibration curve. A separate calibration curve was also prepared



for glucose with the range going up to 60 mg/ ml, along with a CVS of 25 g/l as the glucose content was expected to be much higher. These were then syringe-filtered into labelled vials, sealed and along with the samples and SRS, analysed by HPLC for sugar content.

The following calculations were then performed:

First, the integrity of the calibration curve was verified with a sugar from a different source following this calculation (Equation 4.2):

$$\text{CVS recovery (\%)} = \frac{\text{concentration detected by HPLC (mg/ml)} \times 100}{\text{known concentration of standard (mg/ml)}} \dots\dots\dots \text{(Equation 4.2).}$$

After the hydrolysis, the sugars in the sugar recovery standards (SRS) were then determined to account for any degradation thus:

$$\text{Recovered sugar, \% } R_{\text{sugar}} = \frac{\text{conc. detected by HPLC (mg/ml)}}{\text{known conc of sugar before hydrolysis (mg/ml)}} \times 100 \dots\dots \text{(4.3).}$$

The amount of sugar degradation calculated for each sugar was then used to correct the amount obtained from HPLC analysis of the hydrolysis thus:

$$\text{Corrected conc. of sugar, } C_{\text{corr sample}} = \frac{\text{conc detected by HPLC} \times \text{dilution factor}}{\% R_{\text{sugar}} / 100} \dots\dots\dots \text{(4.4)}$$

Where:  $C_{\text{HPLC}}$  = conc. of a sugar as determined by HPLC, mg/ml %

$R_{\text{sugar}}$  = average recovery of a specific SRS component

$C_{\text{cor. sample}}$  = concentration in mg/ml of a sugar in the hydrolyzed sample after correction for loss on 4% hydrolysis

The total glucan content – Starch glucan content = Cellulose glucan content (Sluiter et al., 2010).

After deducting the amount of starch glucan (**Section 3.2.2.2**) from the total glucan, the concentration of polymeric sugars were calculated from the corresponding monomeric sugars using anhydro corrections of 0.88 for xylose and arabinose (to determine hemicellulose) and 0.90 for glucose and mannose (to determine cellulose) as follows:

$$C_{\text{anhydro}} = C_{\text{corr}} \times \text{Anhydro correction} \dots \dots \dots (4.5).$$

The amount of individual sugars were then calculated thus:

$$\text{Sugar (\%)} = \frac{C_{\text{anhydro}} \times V_{\text{filtrate}}}{\text{ODW}_{\text{sample}}} \dots \dots \dots (4.6)$$

The sum of all the sugars as calculated from equation 4.5 were then recorded as total carbohydrate content (Sluiter et al., 2008).

#### - Lignin Content

This was based on an amended acetyl bromide method (Fukushima and Hatfield, 2001).

#### **Standard preparation**

A standard lignin solution was prepared by dissolving 10 mg of lignin (Sigma Aldrich, UK) in 5 ml of 80 % dioxane. Various quantities, from 0.2-0.5 ml of lignin-dioxane solution, were transferred to fresh tubes containing 0.5 ml of 25% acetyl bromide in glacial acetic acid and incubated at 50°C too for a period of 30 min, exactly 90 min after the samples were placed in the bath. Both samples and standards were thus ready after 2 hr. All experiments were performed in triplicates.

A blank solution was also prepared of 0.2 ml dioxane in 0.5 ml of the solution of 25%v/v acetyl bromide in glacial acetic acid.

## Sample

Into 50 ml Teflon-capped Pyrex<sup>®</sup> culture tubes, 100 mg of bran was transferred and 4 ml of 25% v/v solution of acetyl bromide in glacial acetic acid was added. The tubes were closed tight with the screw caps and incubated in a water bath at 50°C for 2 hours. The tubes were left to cool for 5 minutes before dilution to 16 ml by the addition of 12 ml glacial acetic acid. They were then centrifuged at 300 rpm for 15 min and 0.5 ml of the samples were then transferred to fresh tubes and made up to 10 ml according to the formula in **Table 3.1** below.

**Table 3.1:** Preparation of samples for lignin determination

<b>Solution</b>	<b>Sample (ml)</b>	<b>Standard (ml)</b>
Quantity of sample	0.5*	0.7-1.1
Glacial acetic acid	2.5	2.5
0.3M NaOH	1.5	1.5
0.5M hydroxylamine HCl	0.5	0.5
<b>Subtotal</b>	5.0	5.2-5.6
Glacial acetic acid to add	5.0	4.8-4.4
<b>Total</b>	10.0	10.0

\*: 0.5 g of bran was estimated to have a volume of 0.5 ml

After the solutions were prepared as above, the sample tubes and the standard were mixed using a vortex mixer and then pipetted into a quartz cuvette and analysed using a Jenway 7315 spectrophotometer (Bibby Scientific Ltd, UK) at 280 nm.

A calibration curve was drawn by plotting the absorbance against the concentration of lignin in the standard solutions and the amount in samples determined by interpolation from the regression equation of the curve.

#### - Protein Content Analysis

Protein content was determined using the Thermo Scientific Flash EA1112 N/Protein Analyser which estimates protein based on a complete combustion of the sample within a high temperature reactor, and the determination of the elemental gases produced. The test was performed by technical staff in the North Lab (School of Biosciences, Sutton Bonington, University of Nottingham) where the elemental analyser was set up, according to their in-house methodology. The brans were ground to as fine a powder as possible, then 50 mg each of samples in triplicate, blanks & standards were weighed into foil capsules and sealed & flattened. The blank used was sucrose (no nitrogen N content) and the standard used was aspartic acid, which is known to be 10.54% nitrogen and all samples were then placed in the auto-sampler carousel. The % N figure obtained was converted to protein by multiplying by 6.25.

#### - Lipid Content

The total lipid content was determined by gas chromatography by the direct fatty acid synthesis method in the North Laboratory (School of Biosciences, Sutton Bonington, University of Nottingham). To labelled methylation tubes 1.0 g of ball milled bran samples, 0.7 ml of 10 M potassium hydroxide and 5.3 ml of methanol were added. The tubes were incubated at 55°C and vortexed at 20 min intervals for 90 min before being transferred to a cold water bath for 10 min to cool. Next, 0.58 ml of 12 M sulphuric acid was added to the samples, vortexed and the incubation and cooling steps repeated. Next, 3 ml of hexane was added to the samples and each tube vortexed for 30 sec then centrifuged at 1,500 rpm for 5 min. The top fatty acid-hexane layer was then transferred to labelled LP4 tubes and dried down by blowing nitrogen through them. The residual fatty acids were then resuspended in 300 µl hexane, vortexed and transferred to solvent-resistant Eppendorf tubes from which 100 µl was aliquoted into vials with inserts and analysed by gas chromatography by technical staff of the North Laboratory (School of Biosciences, Sutton Bonington, University of Nottingham).

### **3.2.2.2 Other Compositional Analyses**

In addition to the above analyses which comprise the bulk of a summative mass closure, the following sub-components were specifically determined in order to get a clearer picture of the biomass as described below.

#### **- Starch Content**

The starch content was determined specifically as the sorghum bran biomass is a grain-based one. It was determined using the test kit by Megazyme® (Ireland) according to manufacturer's instructions using method ("c") outlined for the determination of total starch in samples containing resistant starch but no D-glucose and/or maltodextrins. All solutions and enzymes were prepared/diluted according to the instructions leaflet.

The brans were first milled to pass a 0.5 mm screen then 100 mg weighed accurately to 0.1 mg was transferred to a 16 x 120 mm glass tube. The samples were wetted with 0.2 ml of 80 % ethanol to aid dispersion, and then stirred on a vortex mixer. Small stirrer bars were placed in the tubes and the tubes were placed in an ice bath then the entire set up was placed on a magnetic stirrer plate. Two millilitres of 2 M KOH was added as the mixture was stirred then the samples were left for about 20 min to re-suspend the pellet and dissolve the resistant starch. Next 8 ml of 1.2 M sodium acetate buffer pH 3.8 was added to the tubes followed immediately by 0.1 ml of thermostable  $\alpha$ -amylase (~1,000 U/ml) and 0.1 ml of amyloglucosidase (~3,300 U/ml) and the tubes transferred from stirrer to a bath at 50°C. The samples were incubated for 30 min with intermittent mixing then transferred quantitatively to a 100 ml flask and adjusted with water to 100 ml. An aliquot was then centrifuged at 1,800 g for 10 min.

Duplicate 0.1 ml aliquots of each sample, glucose controls and reagent blanks (0.1 ml water) were transferred to the bottom of glass tubes and then 3.0 ml of glucose oxidase/peroxidase (GOPOD) reagent added. All tubes were incubated at 50°C for 20 min and absorbance read at 510 nm against the reagent blank. Readings were then converted to glucose concentration by comparing with a standard containing 0.1 ml of 1 mg/ml glucose standard solution and 3.0 ml of GOPOD. The glucose content was converted to starch according to the calculations in the manual, notably using the conversion factor of glucose to starch, 0.9 (According to instructions in the Megazyme starch test kit manual.)

- Structural Carbohydrates Content

The cellulose content was estimated by deducting the starch glucose content for each sorghum bran from the total carbohydrate glucose and mannose content and multiplying by 0.9. The hemicellulose content was determined by multiplying the xylose and arabinose content by 0.88 (Sluiter et al., 2008).

Starch glucan x 1.1 = glucose content attributable to starch

Glucose content attributable to cellulose = Total glucose estimated – glucose content attributable to starch

Cellulose content = (Glucose content attributable to cellulose + mannose) x 0.9 .....(4.9)

Hemicellulose content = (Xylose and arabinose content) x 0.88 .....(4.10)

- Reducing Sugar Analysis

The reducing sugar content of the brans was determined by the DNS method of Miller (1972). Exactly 100 mg of bran was extracted twice with 5 ml of hot 80 % ethanol. The supernatant was evaporated in a water bath at 80°C and 10 ml of water added to dissolve the sugars. Into labelled test tubes, 0.5 to 3.0 ml of this solution was pipetted and equalized to 3 ml with reverse osmosis water. Next, 3 ml of DNS reagent was added and the tubes heated in a boiling water bath for 5 min. The tubes were left till just warm, then 1 ml of 40 % Rochelle salt solution was added (**Section 3.2.1.1**). Upon cooling, the intensity of the dark red colour generated was measured at 510 nm. A series of standards from 0 – 100 mg/l were also analysed and used to plot a standard curve.

### 3.2.3 Generation of Hydrolysate

This section describes the degradation of the carbohydrate component of the bran into the component sugars, mainly glucose, following two methods. In preparing hydrolysates from conventional lignocellulosic materials, there is a prehydrolysis/pretreatment stage using mainly dilute acid (or other methods) in which the hemicellulose component is broken down into a mixture of pentoses and oligosaccharides. Then in the main hydrolysis step, the cellulose is subsequently broken down enzymatically or using acids leaving residual lignin

behind as a solid by-product (Olsson and Hahn-Hägerdal, 1996). However, with a starch-rich substrate, the dilute acid method alone can digest the starchy component into glucose or alternatively, amylolytic enzymes can be used to produce a hydrolysate (**Section 3.1.2**).

This section thus describes the use of both methods for the production of a fermentable sorghum bran hydrolysate. All hydrolysates were vacuum-filtered using Whatman No 1 filter paper and a Buchner funnel. The residues were discarded while the resulting liquid fractions were stored at 4°C pending use.

### **3.2.3.1 Enzyme Hydrolysate**

Hydrolysates were prepared by the enzymatic digestion of the brans based on an adaptation of the technical bulletin of the starch assay kit (Sigma Aldrich, UK). Into duplicate 1 L Duran bottles, 200 g of bran was transferred and 700 ml of water added immediately followed by 100 ml of DMSO. The mixture was vortexed and transferred to a water bath at 95°C, with shaking at 140 rpm for 10 min to gelatinize it, then 50,000 units of porcine pancreatic amylase (Sigma Aldrich, UK) was added. The slurry was incubated for another 10 min then cooled to 50°C in a water bath in 0.2 M sodium acetate buffer at pH 6.9 added. The slurries were vortexed vigorously every 2 min for 6 min then 160,000 units of amyloglucosidase from *Aspergillus niger* in sodium acetate buffer pH 4.5 added. The reaction was left to proceed for 40 min then the volume was adjusted to one litre and reacted for a further 24 hr. Samples were collected at intervals, diluted appropriately and analysed for glucose content by HPLC as described in **Section 3.2.6**.

Glucose yields from starch hydrolysis were calculated thus:

1.0 g of starch + water  $\longrightarrow$  1.11 g of glucose (Borglum, 1980).

Since:

$$\text{Starch digested (g)} = \frac{\text{Amount of bran digested (g)} \times \text{Bran starch content (g)}}{100}$$

So:

$$\text{Theoretical maximum yield (g)} = \text{Starch digested (g)} \times 1.11 \dots \dots \dots (4.7)$$

The hydrolysate was stored at 4°C pending use.

### **3.2.3.2 Dilute Acid Hydrolysate Optimisation**

Optimisation experiments were conducted to determine the optimum acid pretreatment conditions for the production of sugar-rich hydrolysates. In particular the catalyst (mineral acid) type and concentration, and residence time were investigated. Sulphuric acid and nitric acid were investigated at concentrations of 1% and 3%. In triplicates, 500 mg of bran was transferred to Pyrex tubes and made up to 10 ml with the different concentrations of each mineral acid and vortexed. The samples were then digested in an autoclave at 121°C and 15 psi for 15 or 30 min after which the slurry obtained was filtered, through Whatman No 1 filter paper with a Buchner funnel, diluted appropriately and analysed for reducing sugars content by HPLC as described in **Section 2.2.6**.

The sugars released were expressed as a percentage of the sample digested thus:

Amount of sugar A released (mg) = Conc. of A detected by HPLC (mg/mL) x 10mL

To express as a percentage of the bran sample tested:

$$\frac{\text{Amount of sugar A released (mg)} \times 100}{500 \text{ mg}} \dots\dots\dots(4.8)$$

### **3.2.3.3 Solid Loading Ratio Optimisation**

Using the optimum dilute acid pretreatment type and concentration obtained from the last section, the effect of sorghum bran loading on the efficiency of sugar release during pretreatment was investigated.

In triplicates, 0.5 g, 1 g and 2 g of bran were measured into Pyrex glass tubes and made up to 10 ml with the selected concentration of dilute acid to obtain final concentrations of 5 %, 10 % and 20 % w/v bran loading. The samples were then digested in the autoclave at 121°C and 15 psi for 15 min, the resulting slurry filtered through Whatman No 1 filter paper with a Buchner funnel, diluted appropriately and analysed for reducing sugars content by HPLC as described in **Section 3.2.6**.



### 3.2.4 Inhibitory Compound Analysis

The presence of common inhibitors of ethanol fermentation which could be native to, or be produced during hydrolysate preparation was investigated in the bran hydrolysates. Standard solutions containing 0.125-1 g/L of syringaldehyde, acetic acid, hydroxymethylfurfural (HMF), furaldehyde, ferulic acid and vanillic acid were prepared in reverse osmosis water and analysed by HPLC. The sorghum bran hydrolysates were then analysed for these inhibitors.

The HPLC system (Waters Limited, UK) was fitted with a Techsphere ODS-2 column, Photodiode Array detector, a pump and computer system. Hydrolysate samples were prepared by filtering them through a 0.2  $\mu\text{m}$  syringe filter into a vial. An injection volume of 10  $\mu\text{l}$  of the samples were then run by a gradient method using a mixture of neat methanol and a 1:1 methanol:water mixture at a flow rate of 0.5 ml/min. Data was acquired with the Empower<sup>TM</sup> PDA Pro software (Waters, UK) and standard curves were used to estimate inhibitor concentrations.

## 3.3 Results

### 3.3.1 Compositional Analysis

As discussed in **Section 3.1** it is important to determine the composition of the proposed substrate prior to bioconversions as this will provide an insight into the suitability of the feedstock for bioconversion purposes. The composition of sorghum bran obtained from the wet-milling process was investigated as no comprehensive data could be found in the scientific literature. The summative mass closure approach of the National Renewable Energy Laboratory (NREL) with the goal of breaking down a biomass sample into constituents that sum up to 100% by weight was adopted for this research (Sluiter *et al.*, 2010). In addition to the key components determined for this biomass, certain sub-components were determined in order to get a clearer picture of the composition of the biomass.

Results are presented below of the compositional analyses of the white and red sorghum brans as averages and their standard deviations indicated. One-way analysis of variance (ANOVA) was performed on the resulting data for each parameter based on the null hypothesis ( $H_0$ ) that the various parameters are the same in the both brans.

### 3.3.1.1. Moisture Content

The first parameter to be determined was the moisture content (MC) values of the red and white brans, which was determined with a halogen moisture analyser. This is a thermogravimetric method where MC is determined by the direct weight lost by the sample due to the heating generated by intense halogen light.

**Table 3.2:** Moisture content values of the sorghum brans as determined with a halogen moisture analyser. Values are presented as means of triplicate analyses with the standard deviations

SAMPLE	MOISTURE CONTENT (%)
WHITE BRAN	6.81 ± 0.21*
RED BRAN	6.29 ± 0.17*

\* = significantly different values.

It was observed that the moisture content was originally high in the white bran at 11.55% (results not shown) so it was oven-dried at 40 °C until the MC was lowered to a more acceptable value to be 6.81% (**Table 3.2**) and then stored. It was observed that the differences in these MC values were statistically significant according to a Student's t-test ( $P < 0.05$ ;  $t = 0.0299$ ).

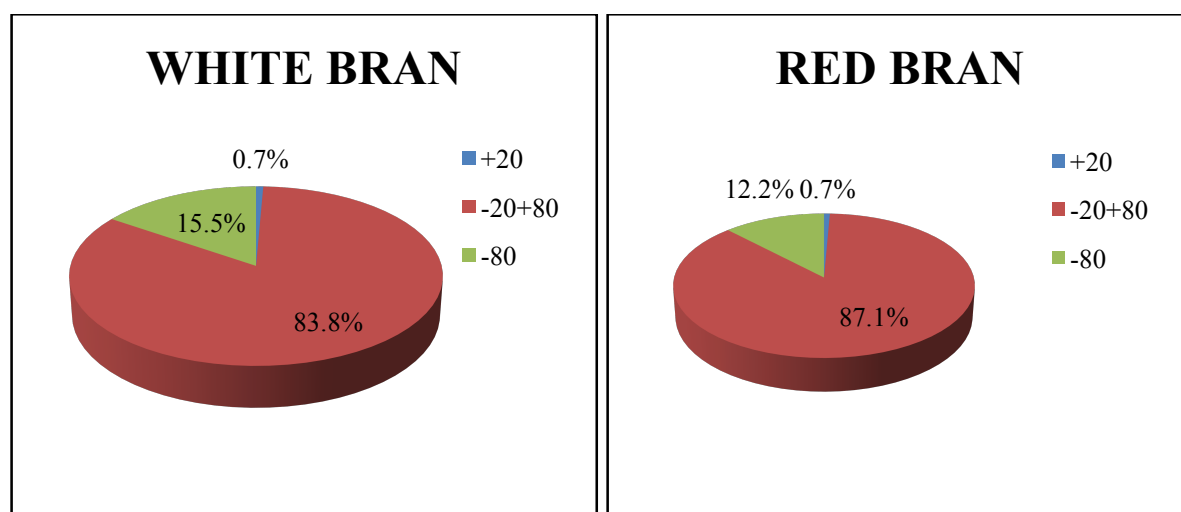
### 3.3.1.2 Sample Preparation

Some preparatory steps were performed in order to get the biomass material into the recommended physical condition. The brans were received in a lumpy state, so they were milled in a planetary ball mill as described in **Section 3.2.2.1** and the effect of the milling is shown in **Figure 3.2** below.



**Figure 3.2:** White sorghum bran samples. Left: As obtained after extraction from wet-milled sorghum grains and air-dried; Right: After milling with the ball mill.

The milled brans were then separated into fractions by sieving using brass sieves. The results are presented as a pie chart in **Figure 3.3** below.



**Figure 3.3:** Composition of the milling fractions obtained by sieving milled sorghum bran

It was observed that a high proportion (12.2% – 15.5%) of the ball-milled brans passed through the 80-mesh sieve. Sieving may cause some components of a feedstock to concentrate in the fines (- 80) fraction, causing a change in composition (Sluiter *et al.*, 2010).

Considering that sorghum bran is a heterogeneous material it was thus determined that the -20/+80 fraction alone may not be completely representative of the bran as sieving could interfere with representative sampling. Considering also, that the +20 fraction was almost insignificant in quantity, it was decided to utilise the milled brans without sieving.

### 3.3.1.3 Total Ash Content

Ash refers to the inorganic residue left over when organic components such as starch and protein are burnt off, and contains the mineral component of the biomass. The glumes are readily removed from the sorghum grain head by threshing and winnowing during post-harvest processing thus resulting in a low ash content (Wall and Blessin, 1970). The findings of this investigation are presented below (**Table 3.3**).

**Table 3.3:** The ash content as % w/w of both sorghum brans. Values are presented as means of triplicate analyses with the standard deviations.

SAMPLE	ASH CONTENT (%)
WHITE BRAN	1.83 ± 0.25*
RED BRAN	1.45 ± 0.19*
* = significantly different values.	

The ash contents of both brans were found to be significantly different according to a t-test ( $P < 0.05$ ;  $t = 0.0000$ ;  $df = 3$ ).

### 3.3.1.4 Carbohydrate Content

The structural carbohydrate content of both sorghum brans (% w/w) was determined by the two-step acid hydrolysis method which hydrolyses biomass matrix carbohydrates into solution which are then quantified by HPLC. However, because sorghum bran is a biomass from a starch-rich grain, this was taken into consideration and the values from the test for structural carbohydrates was adjusted to exclude the glucose contribution from starch. Thus the glucose content obtained from this test was assumed to come from both structural

carbohydrates and starch (**Table 3.4**). There was no statistically significant difference in the total sugar content ( $t = -0.2774$ ;  $P = 0.8075$ ), or individual sugar components, between the different white and red sorghum brans.

**Table 3.4:** Carbohydrate content of the sorghum brans expressed (% w/w) as the detectable sugars. Values are presented as means of triplicate analyses with the standard deviations.

SUGAR COMPONENT	AMOUNT (%) <sup>*</sup>	
	WB	RB
GLUCOSE	57.96 ± 5.07 <sup>a</sup>	59.87 ± 2.14 <sup>a</sup>
XYLOSE	2.96 ± 0.08 <sup>b</sup>	3.05 ± 0.05 <sup>b</sup>
ARABINOSE	3.13 ± 0.89 <sup>c</sup>	2.88 ± 0.05 <sup>c</sup>
MANNOSE	4.98 ± 0.28 <sup>d</sup>	4.69 ± 0.21 <sup>d</sup>
TOTAL SUGARS	69.03 <sup>e</sup>	70.48 <sup>e</sup>

\* Values with same superscript on a row are not significantly different ( $P < 0.05$ )

WB = White Bran, RB = Red Bran.

### 3.3.1.5 Lignin Content

Lignin is the second most abundant naturally occurring polymer, and is a highly cross-linked heteropolymer made of three constituent substituted phenols namely coniferyl, sinapyl, and *p*-coumaryl alcohols and it has been shown to be present in various compositions in cereal grains (Bunzel *et al.*, 2004) where they serve to provide mechanical resistance (Moreira-Vilar *et al.*, 2014). These phenolic compounds could potentially act as inhibitors of fermentation, and their presence in large amounts could pose a problem to the bioconversion process.

The lignin content was determined by the acetyl bromide method which is based on the solubilisation of lignin under acidic conditions and the results obtained are presented in **Table 3.5** below.

**Table 3.5:** The lignin content of both sorghum brans (% w/w) as determined by the acetyl bromide method. Values are presented as means of triplicate analyses with their standard deviations.

SAMPLE	LIGNIN (%)
WHITE BRAN	6.73 ± 0.64*
RED BRAN	10.53± 0.81*

\* = significantly different values at P<0.05

The lignin content of the red bran was found to be significantly higher than that in the white bran., at 10.53% and 6.73% respectively, according to a Student's t-test (df=1; t = -8.8427; P = 0.0009).

### **3.3.1.6 Protein Content**

The protein content of the brans were assayed to determine their potential suitability for bioconversions, particularly without requiring nitrogen supplementation. A Student's t-test of the means of the values obtained indicate that the protein content of the red bran was significantly higher (t = -3.3462; d.f. = 4; P = 0.0287) than that of the white bran at P < 0.05 (**Table 3.6**).

**Table 3.6:** Protein content (% w/w) of both sorghum brans as determined by gas chromatography. Values are presented as means of triplicate analyses with the standard deviations.

SAMPLE	Protein (%)
WHITE BRAN	15.61 ± 0.56*
RED BRAN	16.94 ± 0.13*

\* = significantly different values at P<0.05

### 3.3.1.7 Lipid Content

Sorghum like most cereals has a relatively low lipid content, but this will still contribute to the summative mass closure. Lipids are found in various fractions of starch wet-milling process including the germ, starch and the gluten fractions (Baldwin and Sniegowski, 1951) with over 75 % of the kernel oil located in the germ fraction (FAO, 1995). Lipid analysis of sorghum brans was performed by the saponification of any triacylglycerols into free fatty acids and glycerol, and the subsequent methylation of the fatty acids in to fatty acid methyl esters (FAMES). The concentration of the FAMES was then determined by GC. It was observed that the lipid contents of the brans were low (**Table 3.7**).

**Table 3.7:** Lipid content as % w/w of both sorghum brans as determined by gas chromatography. Values are presented as means of triplicate analyses with the standard deviations.

SAMPLE	LIPID CONTENT (%)
WHITE BRAN	2.32 ± 0.09*
RED BRAN	3.66 ± 0.13*

\* = significantly different values at P<0.05

The values showed that the RB had a statistically higher lipid content than WB according to a T-test (  $t = -20.5412$ ;  $df = 4$ ;  $P = 0.000$ ).

### 3.3.1.8 Summary Of Summative Mass Closure

The total obtained from the various analyses conducted are presented below (**Table 3.8**).

**Table 3.8:** Summative mass closure of the white and red sorghum brans employed in bioconversion processes in this work.

COMPONENT	WHITE BRAN (%)	RED BRAN (%)
MC	6.81 <sup>a</sup>	6.29 <sup>b</sup>
Ash	1.83 <sup>c</sup>	1.45 <sup>d</sup>
Carbohydrate	69.03 <sup>e</sup>	70.48 <sup>e</sup>
Lignin	6.73 <sup>f</sup>	10.53 <sup>g</sup>
Protein	15.61 <sup>h</sup>	16.94 <sup>i</sup>
Lipid	2.86 <sup>j</sup>	3.66 <sup>k</sup>
<b>TOTAL</b>	<b>102.87</b>	<b>109.35</b>

<sup>a,b</sup> Values with different superscripts on a row are significantly different at  $P < 0.05$ .

### 3.3.2 Other Compositional Analyses

Other analyses were performed in order to provide a clearer picture of the composition of the feedstock. The carbohydrate content for instance was further investigated to determine the types of carbohydrates that it was comprised of. To this end, starch analysis and the dinitrosalicylic acid test for reducing sugars were performed.



### 3.3.2.1 Starch Content

Sorghum has a high starch content which is extracted for food applications. The extraction processes employed vary in efficiency and the residual starch content could be high depending on the method used. For instance, the sorghum bran obtained from a decortication process was found to still contain 30% starch (Corredor et al, 2007). Considering that sorghum is the only cereal that contains starch in the pericarp (Schober and Bean, 2011) it is thus important to know the true starch content of the bran.

The starch contents of both brans were determined using the Megazyme® starch determination test kit which uses thermostable  $\alpha$ -amylases and amyloglucosidase to determine starch content based on the ability of  $\alpha$ -amylases to hydrolyse starch into soluble maltodextrins. Resistant starch which is not easily solubilised is pre-dissolved in appropriate solvents, then solubilised; amyloglucosidase then quantitatively hydrolyses maltodextrins into D-glucose. The glucose is then converted to starch using a conversion factor. The brans were found to contain relatively high amounts of starch, as shown in **Table 3.9** below.

**Table 3.9:** Starch content (% w/w) of the sorghum brans as determined following complete amylase and amyloglucosidase digestion. Values are presented as means of triplicate analyses with the standard deviations

SAMPLE	STARCH (%)
WHITE BRAN	49.66 $\pm$ 0.86*
RED BRAN	52.96 $\pm$ 1.43*

\* Indicates a statistical difference between the values ( $P < 0.05$ ).

A student's t-test confirmed that there were significant differences in the starch content (%) of WB and RB ( $t = -3.9509$ ;  $df = 6$ ;  $P = 0.0075$ ). These high starch contents are indicative of inefficiency in the wet-milling and sieving processes and demonstrate that a large amount of starch is not extracted during the wet-milling process.

### 3.3.2.2 Structural Carbohydrate Content

The total glucose content was then used to determine the cellulose while xylose content was used to estimate hemicellulose content, as described in **Section 3.2.2.1**, after deducting the starch contribution from the total glucose content. It was observed that the content of structural carbohydrates was relatively low at 5.21 % - 5.35 % for hemicellulose and 5.67 – 7.48 % cellulose contents (**Table 3.10**).

**Table 3.10:** Structural carbohydrate content (% w/w) of the sorghum brans. Values are presented as means of triplicate analyses with the standard deviations

COMPONENT	AMOUNT (%)	
	CELLULOSE	HEMICELLULOSE
WHITE BRAN	7.48	5.35*
RED BRAN	5.67	5.21

\* Indicates a statistical difference between the values ( $P < 0.05$ ).

A statistically significant difference was found in the cellulose contents of both brans ( $t = 12.9728$ ;  $df = 4$ ;  $P = 0.0237$ ), while for hemicellulose it was not significant ( $t = 1.1322$ ;  $df = 4$ ;  $P = 0.2829$ ).

### 3.3.2.3 Reducing Sugar Content

The reducing sugar content was investigated by the DNS method to determine if the brans had any free sugars. The DNS test detects sugars that have a free carbonyl group that has the ability to reduce the DNS reagent. These sugars include all monosaccharides such as glucose and fructose, and some disaccharides such as lactose. This test aims to determine their contribution to the total carbohydrate content and the results are shown in **Table 3.11**.

**Table 3.11:** Reducing sugar content (% w/w) of sorghum brans determined by the dinitrosalicylic acid (DNS) method. Values are presented as means of triplicate analyses with the standard deviations.

SAMPLE	REDUCING SUGARS (%)
WHITE BRAN	0.85 ± 0.16*
RED BRAN	1.16 ± 0.07*

\* Indicates a statistical difference between the values ( $P < 0.05$ ).

It was observed that only a negligible amount of free reducing sugars was present in the brans with just around 1% w/w of the bran being made up of free sugars. The values were significantly different, with higher free sugars in the red bran ( $t = -1.6945$ ;  $df = 4$ ;  $P = 0.1654$ ).

### 3.3.3 Hydrolysates Generation

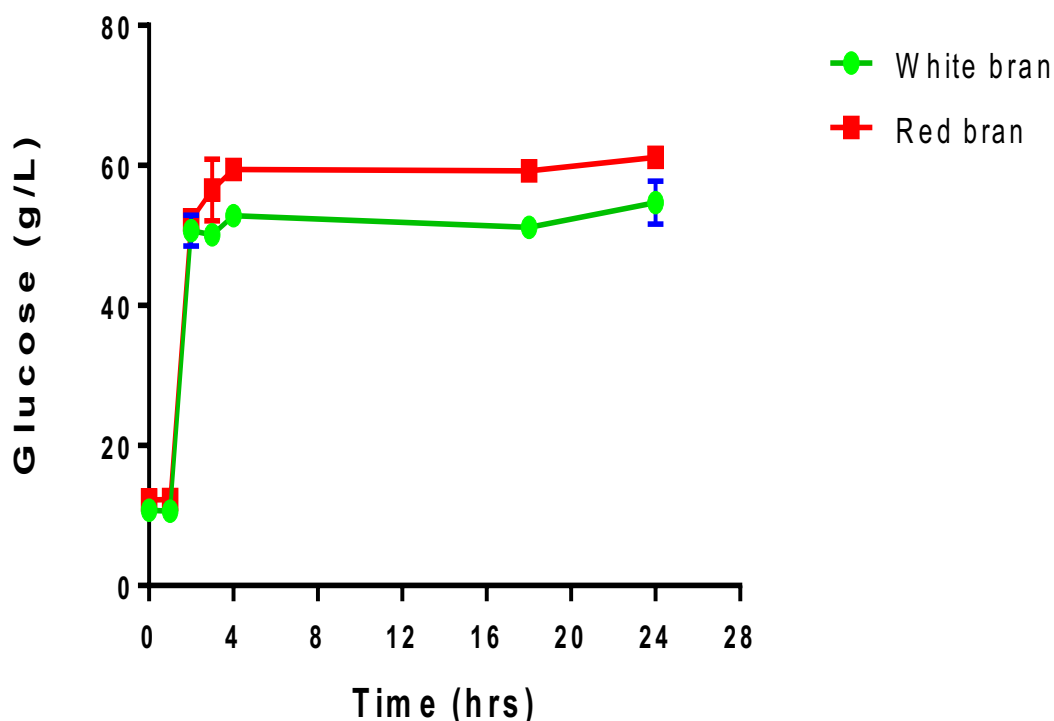
A hydrolysate essentially refers to the product of a hydrolysis process. Hydrolysates are produced from biomass following some form of pretreatment where necessary.

Dilute acid- and enzyme-generated hydrolysates were prepared using the optimum conditions obtained from the dilute acid and enzymatic hydrolyses described in **Section 3.2.3**. The two hydrolysates were investigated to determine the most suitable for downstream bioconversion processes. The detailed findings are presented below.

#### 3.3.3.1 Enzyme Hydrolysate Preparation

Amylolytic enzymes digest starch into maltose, glucose and maltodextrins.  $\alpha$ -amylases are bacterial, fungal or porcine enzymes that hydrolyse 1,4- linkages in both amylose and amylopectin into glucose and maltose. Amyloglucosidases (glucoamylase) are fungal enzymes that catalyse the hydrolysis of maltose into glucose by the 1,3,  $\alpha$ -1,6 and beta-1,6 linkages by primarily cleaving 1,4-linkages of  $\alpha$ -D-glucopyranosyl units of non-reducing ends, releasing Beta-D-glucopyranose (Hobbs, 2009).

Results of the enzymatic conversion of starch in the sorghum bran are shown in this section, and the time course for the production of hydrolysate is shown in **Figure 3.4** below.



**Figure 3.4:** Time course of sorghum bran hydrolysis using amylolytic enzymes to achieve residual starch conversion into glucose. Bran loading rate was 20% and the reaction was held at 50°C for 24 hr, and amount of glucose estimated by HPLC. Values are averages (n = 3), error bars are standard deviations (some error bars are too small to be visible).

It was observed that glucose release from the enzymatic digestion of the red bran peaked at about 61 g/L and plateaued after about 6 hr, similarly the white bran glucose yield was observed to peak at about 55 g/L before similarly plateauing. The residence time for subsequent enzyme hydrolysate preparations was thus set at a maximum of 12 hr.

### 3.3.3.2 Dilute Acid Optimisations

The amount of sugars liberated in the pretreatments using two concentrations of 1 % and 3 % of sulphuric acid and nitric acid in the digestion of red and white brans was investigated at two residence times of 15 and 30 min. The results are presented in **Table 3.12** below.

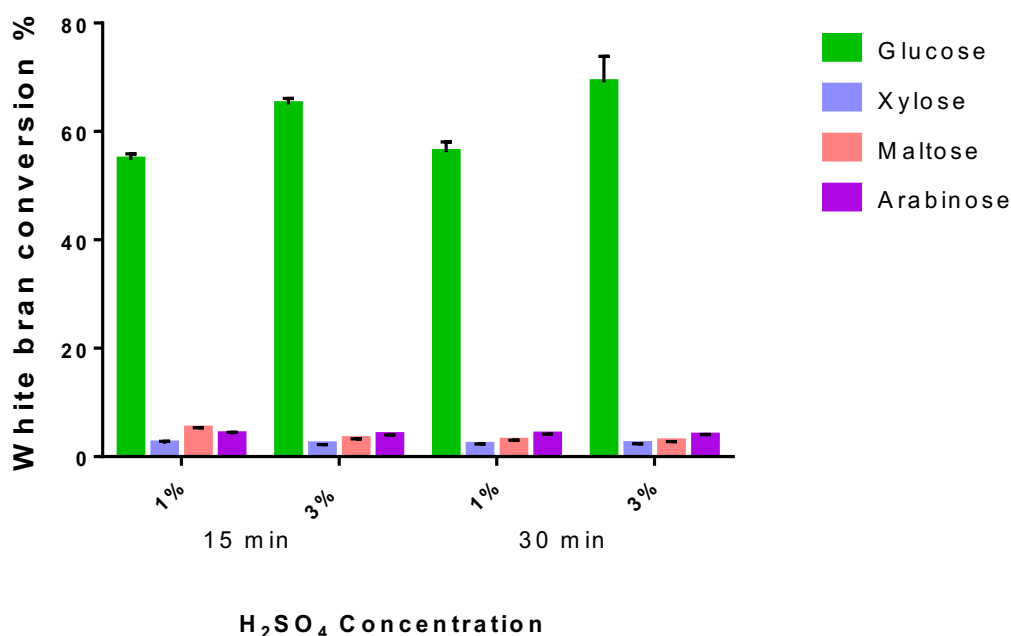
Table 3.12: Sugars released in dilute acid optimisation experiments. Values are averages of triplicate observations with the standard deviations.

sample	Time	Treatment	Concentration (g/L)			
			Glucose	Xylose	Maltose	Arabinose
White bran	15 min	1% H <sub>2</sub> SO <sub>4</sub>	27.37 ± 4.09	1.2 ± 0.21	2.58 ± 0.08	2.04 ± 0.19
		3% H <sub>2</sub> SO <sub>4</sub>	32.24 ± 7.27	1.1 ± 0.02	1.60 ± 0.04	1.96 ± 0.00
	30 min	1% H <sub>2</sub> SO <sub>4</sub>	28.07 ± 0.96	1.06 ± 0.10	1.45 ± 0.07	2.01 ± 0.10
		3% H <sub>2</sub> SO <sub>4</sub>	34.53 ± 8.04	1.15 ± 0.02	1.38 ± 0.02	1.92 ± 0.10
	15 min	1% HNO <sub>3</sub>	25.77 ± 1.15	3.11 ± 0.41	1.12 ± 0.04	2.17 ± 0.40
		3% HNO <sub>3</sub>	30.85 ± 0.69	1.13 ± 0.03	0.72 ± 0.02	1.96 ± 0.00
	30 min	1% HNO <sub>3</sub>	26.34 ± 1.05	2.15 ± 1.57	1.00 ± 0.02	2.13 ± 0.40
		3% HNO <sub>3</sub>	35.25 ± 4.32	5.47 ± 0.01	4.68 ± 0.16	4.85 ± 3.90
Red bran	15 min	1% H <sub>2</sub> SO <sub>4</sub>	26.48 ± 1.06	0.75 ± 0.05	2.22 ± 0.76	1.66 ± 0.10
		3% H <sub>2</sub> SO <sub>4</sub>	32.17 ± 6.05	0.79 ± 0.01	4.18 ± 0.16	2.13 ± 0.60
	30 min	1% H <sub>2</sub> SO <sub>4</sub>	27.11 ± 4.84	0.75 ± 0.00	1.30 ± 0.15	1.95 ± 0.00
		3% H <sub>2</sub> SO <sub>4</sub>	31.68 ± 5.71	4.53 ± 0.32	8.56 ± 0.08	3.63 ± 2.70
	15 min	1% HNO <sub>3</sub>	28.25 ± 0.33	2.51 ± 0.04	1.04 ± 0.04	1.76 ± 0.00
		3% HNO <sub>3</sub>	30.32 ± 0.63	3.08 ± 0.05	1.12 ± 0.02	2.38 ± 0.00
	30 min	1% HNO <sub>3</sub>	26.13 ± 1.52	2.46 ± 0.00	0.81 ± 0.00	1.98 ± 0.00
		3% HNO <sub>3</sub>	29.51 ± 1.05	3.38 ± 0.09	0.48 ± 0.14	2.31 ± 0.10

Similarly, the bran conversion efficiencies into simple sugars at the various conditions are shown in **Figures 3.5 - 3.8**, expressed as a percentage of the amount of sample hydrolysed. It was clear that in all treatments, glucose accounted for most of the reducing sugars liberated during the dilute sulphuric acid digestion of the brans while the levels of xylose, arabinose and maltose produced were relatively low. Importantly, it was observed that the maltose content was low in all treatments which indicates that the digestion of the bran carbohydrates proceeded to completion to glucose and not the maltose disaccharide. Data was analysed by multifactor ANOVA in the relevant sections below.

#### - White Bran Conversion

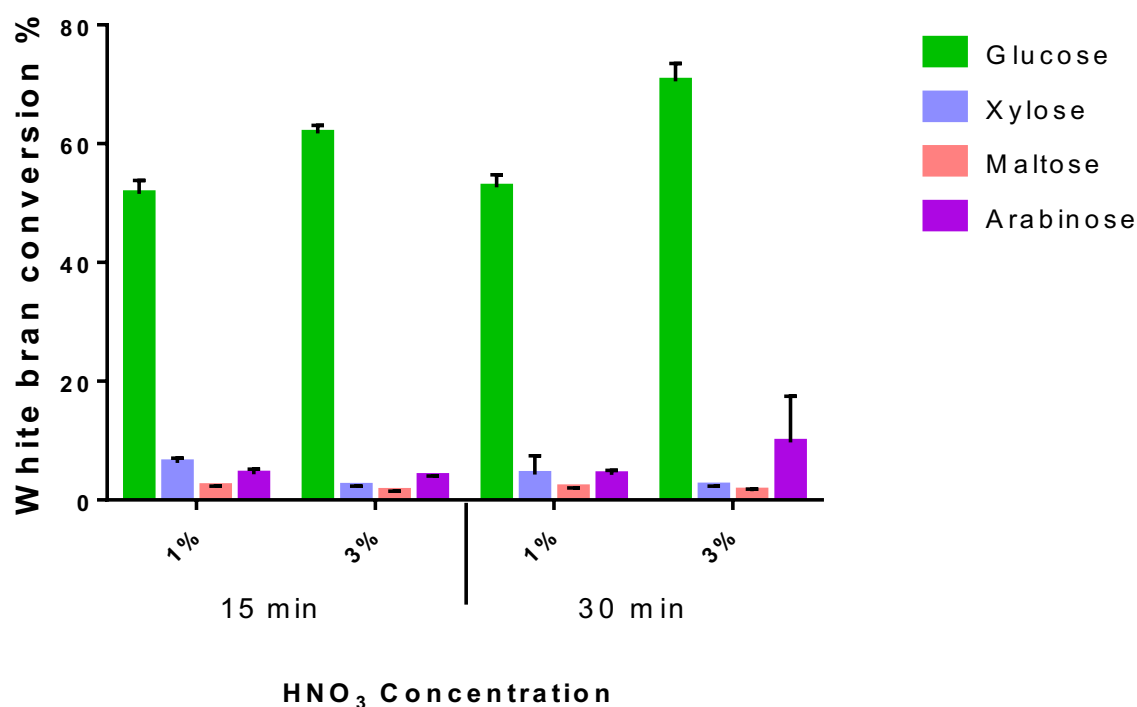
Dilute sulphuric acid effectively converted the WB into various simple sugars (**Figure 3.5**) and it was observed that the best treatment, in terms of sugar release, was the 3 %  $\text{H}_2\text{SO}_4$  digestion for 30 min which liberated 69.1 % of the WB to glucose and between 2.3 % and 3.8 % of the other sugars. Multiple Range Tests for glucose by residence time show no statistically significant differences with the means of values at either the 15 or 30 min times (**Table 3.12**), with approximately 65 % of the bran converted to sugars at 15 min residence time.



**Figure 3.5:** Effect of two concentrations of sulphuric acid on conversion efficiency of WB into reducing sugars during pretreatment for 15 or 30 min at a 5% solids loading rate. Amounts of sugars released are expressed as a percentage of bran hydrolysed. Values are averages ( $n = 3$ ), error bars are standard deviations.

Meanwhile there was no statistical difference between WB conversion at 15 or 30 min when utilising 1 %  $\text{H}_2\text{SO}_4$  (data not shown). Xylose was the least product of the conversion across all treatments and no treatment increased the conversion of the bran to any other sugar besides glucose.

With dilute nitric acid pretreatment of white bran it was observed that glucose yields were significantly higher in all conditions than the other sugars, whose total amounts accounted for a maximum of 15% of the bran conversion (**Figure 3.6**). The highest levels of bran conversion to simple sugars was observed when using 3 %  $\text{HNO}_3$  and a residence time of 30 min, with 70.5% of white bran being converted to glucose and 9.7% to arabinose.

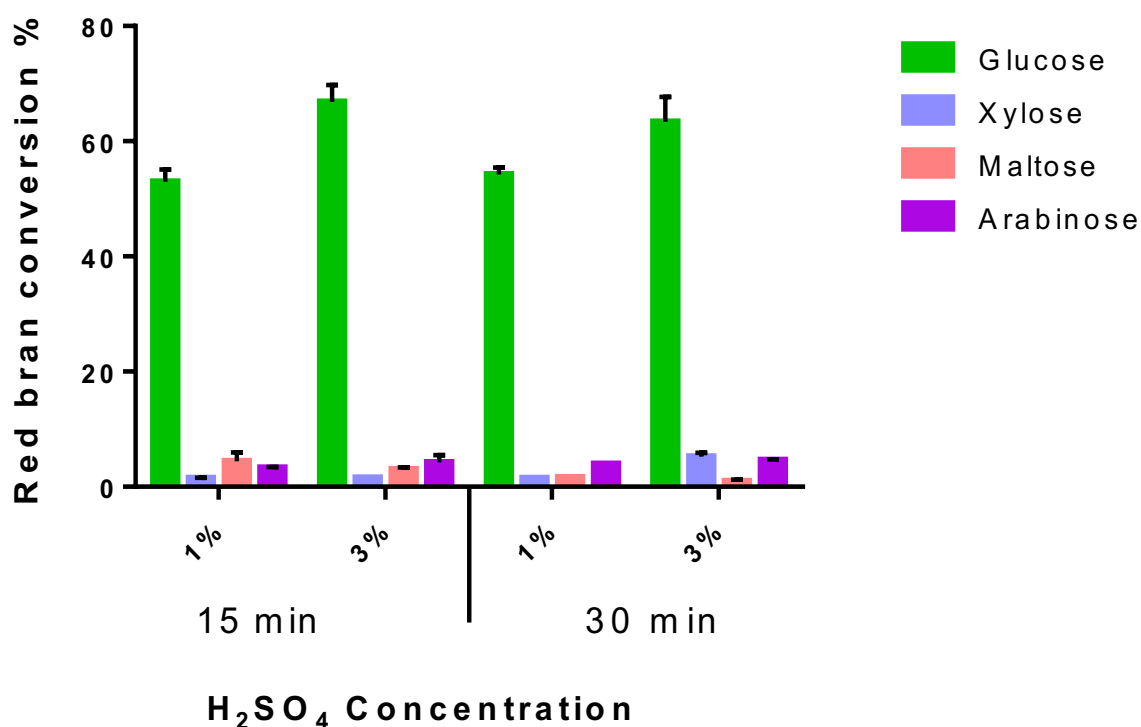


**Figure 3.6:** Effect of two concentrations of nitric acid on conversion efficiency of WB into reducing sugars during pretreatment at  $121^\circ\text{C}$  for 15 and 30 min at a 5% solids loading rate. Amounts of sugars released are expressed as a percentage of bran hydrolysed. Values are averages ( $n = 3$ ), error bars are standard deviations.

It was observed that a maximum amount of arabinose, 4.18 %, was liberated at the most severe digestion conditions of 3% nitric acid and residence time of 30 min, however, this was not significantly higher than the observed conversion efficiency at 15 min digestion where 62.7% and 3.8% of the bran was converted into glucose and arabinose respectively ( $F = 1.05$ ;  $df = 1$ ;  $P = 0.3636$ ). The concentration of acid used had a very significant effect on glucose release ( $F = 76.62$ ;  $df = 1$ ;  $P = 0.0009$ ) while time was less significant ( $F = 9.69$ ;  $df = 1$ ;  $P = 0.0358$ ).

#### - Red Bran Conversion

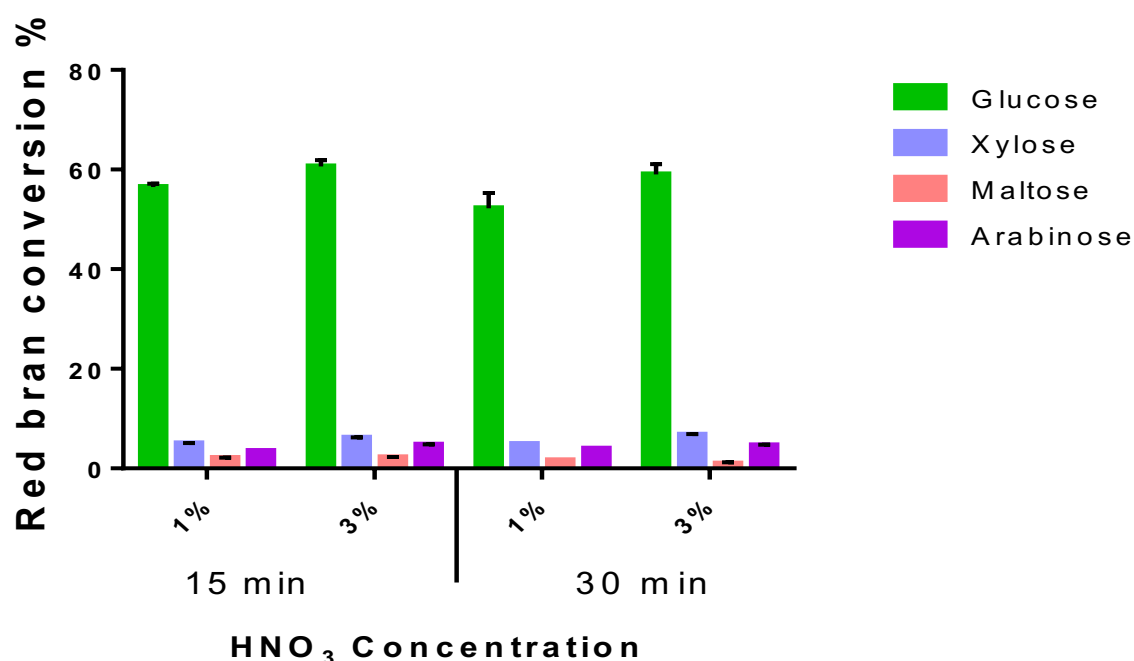
When using dilute sulphuric acid as a pretreatment for red bran, the higher residence time of 30 min resulted in a lower RB conversion as rates dropped from the maximum of 66.8 % to 63.4 % (**Figure 3.7**). However, this difference was not significant ( $F = 31.80$ ;  $df = 1$   $P = 0.6160$ ), although there was a significant difference in effect of acid concentration with the 3% concentration yielding highest amounts of glucose ( $F = 0.29$ ;  $df = 1$ ;  $P = 0.0049$ ). Maltose content was highest in the milder condition and declined with increasing severity which also corresponded with an increase in glucose release thus indicating a more complete hydrolysis.





**Figure 3.7:** Effect of two concentrations of sulphuric acid on conversion efficiency of RB into reducing sugars during pretreatment for 15 and 30 min at a 5% solids loading rate. Amounts of sugars released are expressed as a percentage of bran hydrolysed. Values are averages (n = 3), error bars are standard deviations.

For nitric acid conversion of RB into sugars, it was observed that nitric acid released roughly the same amounts of sugars from the RB across all conditions. However, one-way ANOVA revealed that the 3% preparation achieved significantly higher bran conversion ( $F = 9.83$ ;  $df = 1$ ;  $P = 0.0202$ ) than the 1 % preparation (**Figure 3.8**). Surprisingly, the higher residence time of 30 min with 1 % nitric acid resulted in the lowest rate of bran conversion into sugars and a similar decrease with the 3 % concentration. Changes in glucose concentration with holding time were not significant ( $F = 1.31$ ,  $df = 1$ ;  $P = 0.2957$ ) while the maltose content decreased slightly with increased residence time and arabinose remained relatively static (**Figure 3.8**).

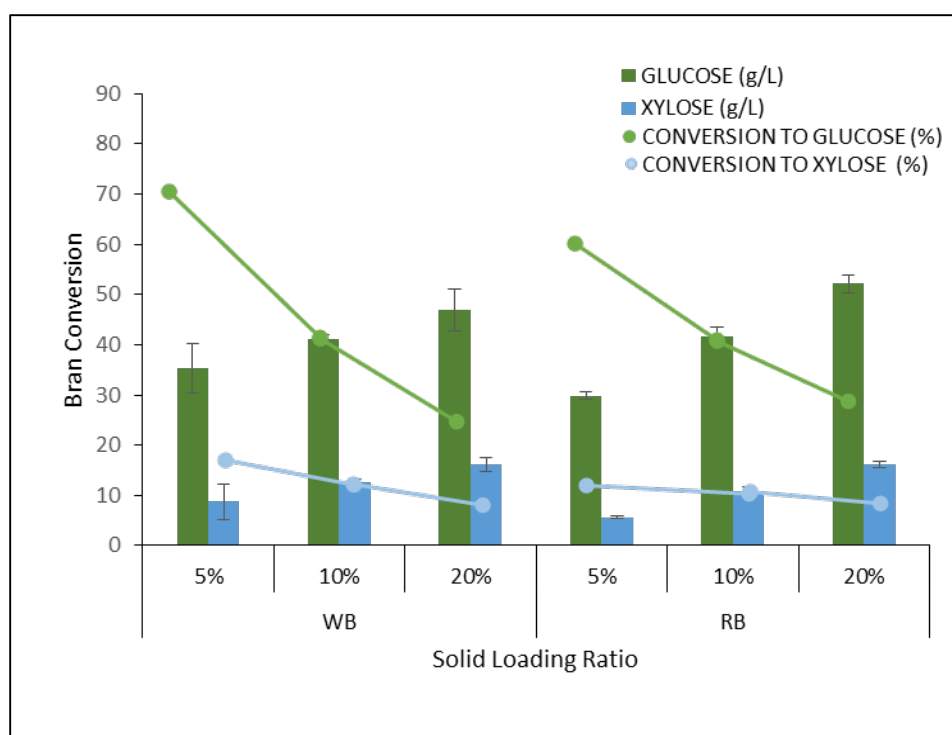


**Figure 3.8:** Effect of two concentrations of nitric acid on conversion efficiency of RB into reducing sugars during pretreatment for 15 and 30 minutes at a 5% solids loading rate. Amounts of sugars released are expressed as a percentage of bran hydrolysed. Values are averages (n = 3), error bars are standard deviations.

In summary, for all the pretreatments and optimisation investigations, none of the pretreatment conditions changed the sugar composition significantly, with glucose being consistently the predominant sugar released while other sugars were mostly only produced in negligible amounts.

### 3.3.2.3 Solid Loading Ratio Optimisation

Having determined the optimum dilute acid pretreatment conditions, the solid loading ratio which represents the total solids content of the bran-dilute acid slurry was then optimised using a multi-factor categorical design. For this experiment only the resulting glucose and xylose amounts are presented (**Figure 3.9**) because other sugars occurred only in negligible quantities.



**Figure 3.9:** The effect of bran loading rate on efficiency of bran conversion into reducing sugars during dilute  $\text{H}_2\text{SO}_4$  hydrolysis at  $121^\circ\text{C}$  for 15 min. The green line represents the proportion of bran (%) converted to glucose during digestion while the blue line represents same for xylose. The green and blue bars show actual concentration of glucose and xylose liberated (g/l) respectively. Values are averages ( $n = 3$ ), error bars are standard deviations.

The bars in **Figure 3.9** show the sugars actually generated at different SL rates (g/L). The null hypothesis was that there would be no difference in the mean sugar outputs of the brans at different solids loading. It was observed that in fact glucose yields increased (in g/L) from 35.4% at 5% SL to 41.2 % and 46.9 % glucose from WB, and from 21.8 to 41.6 % and 52.1 % in RB. Multifactor analyses of variance revealed that there were significant differences between the SL levels ( $F = 51.65$ ;  $df = 2$ ;  $P = 0.0000$ ); and while bran type was not significant in glucose release ( $F = 0.00$ ;  $df = 1$ ;  $P = 0.9741$ ), there was a significant interaction between both factors ( $F = 5.19$ ;  $df = 1$ ;  $P = 0.0238$ ). Multiple range tests show that the mean yields were significantly different in all combinations (**Table 3.13**).

**Table 3.13:** Multiple Range Tests for Conversion to Glucose by SL

<i>Contrast</i>	<i>Sig.</i>	<i>Difference</i>	<i>+/- Limits</i>
10% - 20%	*	16.6223	6.42278
10% - 5%	*	-23.811	6.42278
20% - 5%	*	-40.4333	6.42278

\* denotes a statistically significant difference at  $P < 0.05$ .

With xylose, the amounts released also increased with SL from 8.8 % to 16.1 % and 5.6 % to 16.2 % for WB and RB respectively and these were found to be significant ( $F = 45.61$ ;  $df = 2$ ;  $P = 0.0000$ ). The results showed that there is a significant difference between the glucose and xylose yields (g/L) at different solids loading.

Similarly, the null hypothesis is that the solid loading rate will not result in different bran conversion efficiencies. However, **Figure 3.9** shows that this is not the case as the digestion of both white and red brans followed a similar pattern with the efficiency of the digestion declining with increasing solids loading (SL). For instance, there was a 23.5 % conversion of WB into glucose at 20 % SL, 41.2% at 10 % SL and 70.7 % at 5% SL. This is a 17.7 % and 29.5 % increase respectively as SL decreased. The same trend was found with RB where the 5% SL was most effective and bran conversion to glucose declined by half at 20 % SL. A

multifactor ANOVA showed that these differences were significant ( $F = 137.7$ ;  $df = 2$ ;  $P = 0.000$ ) between all three levels. Solid loading was also found to be significant in conversion of both brans to xylose ( $F = 6.98$ ;  $df = 2$ ;  $P = 0.0098$ ), but a multiple comparison procedure to determine which means are significantly different showed that only conversion at 5% and 20 % levels were significantly different. The interaction between bran type and SL was found to be significant for the conversion to glucose while it was not significant in bran conversion to xylose. The null hypothesis was thus rejected as it was clearly demonstrated that there are significant differences in bran conversion efficiencies at different loading rates.

It is important to note that although there was an increase in the actual amount of sugar liberated with increase in SL, there was nevertheless a decrease in the bran conversion rate as SL increased, and proves that the use of higher SL was less efficient.

#### **3.3.4 Sample Hydrolysates**

Under the optimised conditions of 10 % SL with a 3 % preparation of sulphuric acid as catalyst, experiments were repeated in which hydrolysates of both brans were prepared by digestion at 121°C for 15 min, and results compared to data previously obtained for enzyme hydrolysis (**Figure 3.4**). The dilute acid hydrolysates had lower glucose concentration than the corresponding enzyme hydrolysates in some pretreatment batches, and higher in others. While the yields of the sugars, specifically glucose, in the hydrolysates varied between hydrolysis batches, a typical batch was found to be in the range of the values shown in **Table 3.14** below. It was concluded that the dilute acid hydrolysates were potentially suitable for the subsequent bioconversion processes and that the more costly and demanding enzyme hydrolysis did not offer any major advantage.

**Table 3.14:** Glucose contents of various sorghum bran hydrolysates

HYDROLYSATE TYPE	GLUCOSE (g/L)
White bran enzyme hydrolysate	50.8 - 55.4
White bran dilute acid hydrolysate	48.4 – 52.7
Red bran enzyme hydrolysate	53.1 - 61.2
Red bran dilute acid hydrolysate	51.5 - 57.1

The colour of the generated liquor was dependent on the grain and thus the bran source. The red bran dilute acid hydrolysate (RBDAH) was found to be a deep red colour while the white bran dilute acid hydrolysate (WBDAH) was a dark cream colour. The white bran and red bran enzyme hydrolysates (WBEH and RBEH, respectively) both appeared an off-white to pale cream colour, thus indicating that the dilute acid dissolves the colour forming pigments from the bran into solution, unlike the enzyme pretreatment (**Figure 3.10**).

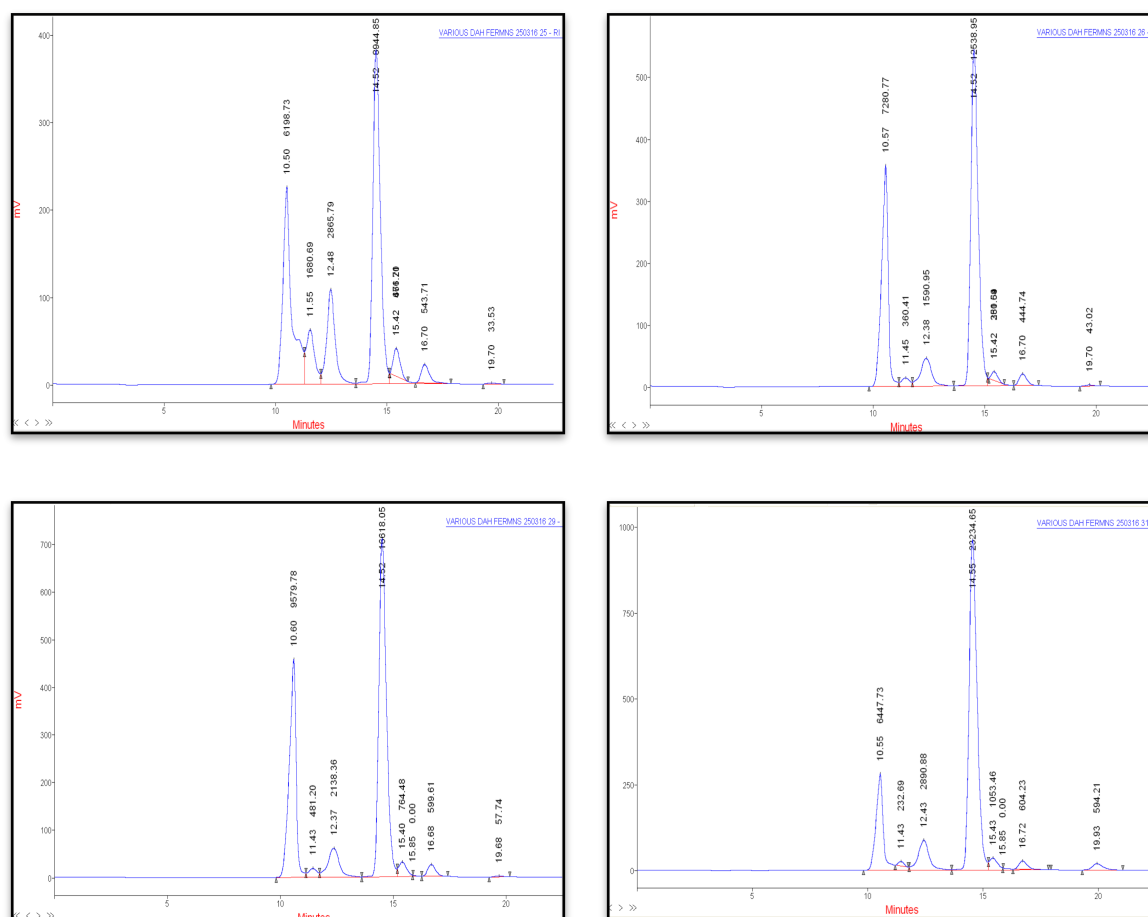


**Figure 3.10:** Left: Sample Hydrolysates showing the appearance of the four hydrolysates prepared from sorghum bran. WBEH = White bran enzyme hydrolysate, WBDAH = White bran dilute acid hydrolysate, RBEH = Red bran enzyme hydrolysate and RBDAH = Red bran dilute acid hydrolysate. Right: 25 ml of RBDAH dispensed into 250 ml Erlenmeyer flasks.

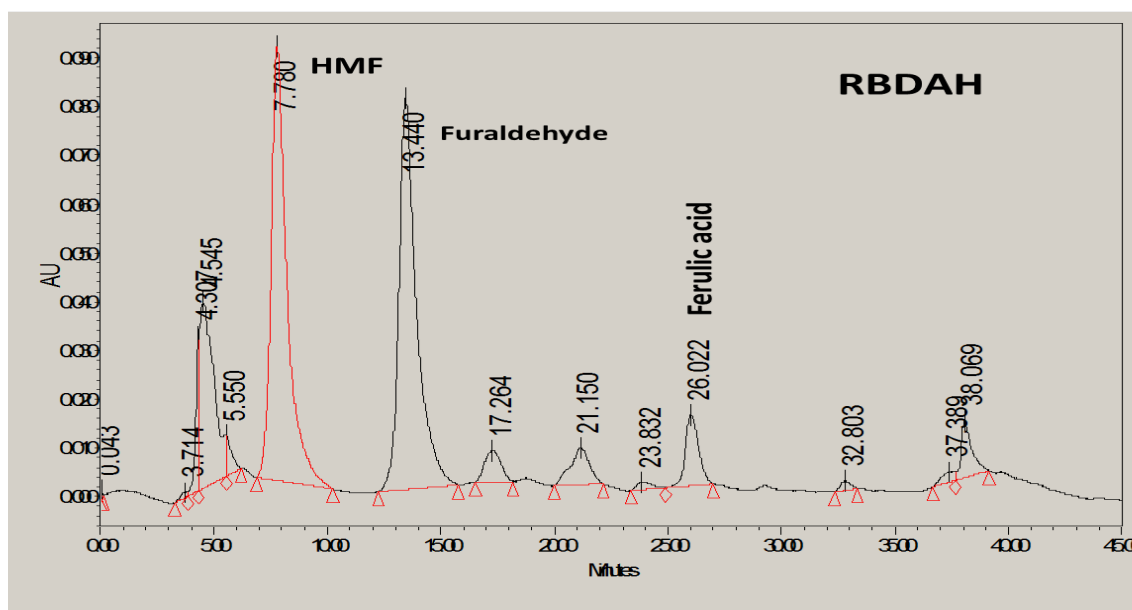
Sample chromatograms of the four hydrolysates as obtained during routine sugar analyses are presented below (**Figure 3.11**).

### 3.3.5 Inhibitor Analysis

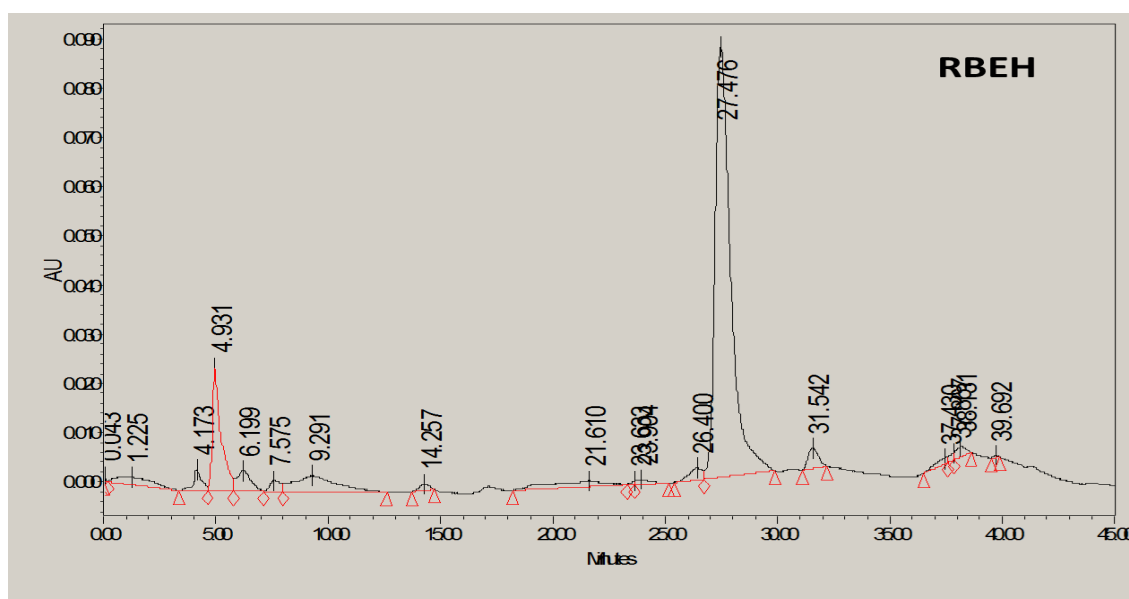
The presence and concentrations of some common inhibitors of fermentation namely syringaldehyde, vanillic acid, ferulic acid, acetic acid, hydroxymethylfurfural (HMF), and furaldehyde were investigated in the various hydrolysates. Knowledge of the nature and amounts of this class of compounds is very important to determine the suitability of a biomass hydrolysate for bioconversion processes (**Section 3.1.3**). The compounds detected in both types of red bran hydrolysates are shown in some representative chromatograms in **Figures 3.12** and **3.13**.



**Figure 3.11:** Chromatograms of the four hydrolysates as obtained from HPLC using a Hi-Plex H<sup>+</sup> column at 45 °C with a mobile phase of 0.005N H<sub>2</sub>SO<sub>4</sub> at flow rate of 0.4 ml/min. *Clockwise:* WBD; RBD; WBE; RBE. Peaks indicate different sugars. *Retention times (r.t. in min.):* Glucose = 14.52 min; Xylose = 15.42; Maltose = 12.42; Arabinose = 16.68. *Note:* Peaks may show slight shifts in r.t. of less than ± 0.1 min.



**Figure 3.12:** Chromatogram showing findings of an investigation of inhibitory compounds in red bran dilute acid hydrolysate (RBDHAH). Hydroxymethylfurfural (HMF), fural and ferulate were detected. HPLC using Techsphere ODS-2 column at room temperature with mobile phase of neat methanol and a 1:1 methanol:water mixture at a flow rate of 0.5 ml/min.



**Figure 3.13:** Chromatogram showing findings of an investigation of inhibitory compounds in red bran dilute acid hydrolysate (RBEH). None of the inhibitors investigated were detected in RBEH. HPLC using Techsphere ODS-2 column at room temperature with a mobile phase of neat methanol and a 1:1 methanol:water mixture at a flow rate of 0.5 ml/min.

It is evident from the chromatograms in **Figures 3.12** and **3.13** that the dilute acid hydrolysate contained more inhibitory compounds than the enzyme hydrolysate given that furfural, HMF and ferulic acids were detected in RBDAH whereas they were not detected in RBEH. The main peak in the RBEH could not be identified. Syringaldehyde was not detected in any hydrolysate. The amounts of the compounds found in the hydrolysates are shown below in **Table 3.15**.

**Table 3.15:** Amounts of various fermentation inhibitors found in a batch of the four hydrolysates. Values are averages of triplicate measurements and the standard deviations.

SAMPLE	CONCENTRATION (mg/l)				
	HMF	FURFURAL	VANILLIC ACID	FERULIC ACID	ACETIC ACID
WBDAH	5.05 ± 0.50	4.27 ± 0.11	5.62 ± 0.91	19.64 ± 1.47	22.37 ± 1.15
WBEH	1.18 ± 0.18	0.33 ± 0.06	ND	1.98 ± 0.21	39.94 ± 3.89
RBDAH	5.93 ± 1.01	5.95 ± 0.71	9.67 ± 1.13	17.12 ± 2.05	30.25 ± 2.76
RBEH	1.04 ± 0.23	ND	ND	ND	42.89 ± 5.42

### 3.4 Discussion

In this chapter, work was undertaken to determine the composition of two types of sorghum bran, namely white sorghum bran and red sorghum bran. Attempts were made to determine the summative mass closure, while other specific components were also determined. In addition, hydrolysates of the brans were prepared using either chemical or enzymatic methods and potential inhibitors elucidated. The findings are discussed in the following sections. It is important to note at the start that all of the data obtained refers to specific samples of white and red bran that were procured at the start of studies and used throughout for consistency. Thus, it is acknowledged that there might be differences should other white



and red bran sources be used. It is also noted at the start of chapter that work focusses on release of sugars from the substrate for later use in fermentations experiments with yeasts and *Aspergillus terreus* to produce value-added products. An alternative approach might have been to have assessed the use of sorghum bran as a solid substrate i.e. solid state fermentation. However, this was considered unlikely to be feasible given that yeasts are not known to grow well on and ferment solid complex lignocellulose sources, and that itaconic acid production by *A. terreus* is favoured by high glucose levels not available in the crude sorghum bran.

### **3.4.1 Compositional Analyses For Summative Mass Closure**

For a feedstock to support microbial growth and bioconversion, it must contain certain essential nutrients which can roughly be divided into: carbon source, energy source, nitrogen source, minerals and vitamins (Nielsen *et al.*, 2012). The summative mass closure gives an indication of the chemical composition of a potential feedstock and this determines the types of bio-conversion processes to which the biomass can be applied.

The moisture contents of the brans were the first parameter determined as these provided the oven dry weight (ODW) upon which the results of other analyses were estimated. It was determined with a halogen moisture analyser which has several advantages over traditional methods including that it employs higher operating temperatures to dry samples and thus has shorter drying times than the traditional oven method. It also uses smaller amounts of sample while retaining its accuracy (while attempting to select the ideal program on the analyser, the results obtained from the barley rap program were compared with a traditional oven method and the differences were found to be less than 5 %). The moisture content of the white bran was found to be higher at about 11.6% than the red bran at 6.3%. This disparity was not surprising because the methods of preparing the bran are not standardised and so could indicate a less vigorous water extraction at the sieving stage, or indicate that it was not dried for as long as the red bran was. As this value was over the 10% benchmark for biomass samples (Sluiter *et al.*, 2010), the white bran was oven dried till the MC was brought to 6.8%. This prevents mould spoilage and also ensures that moisture does not interfere with subsequent compositional analyses as, for instance, high moisture contents could interfere with dilute acid hydrolysis experiments and prevent complete digestion. The bran MC figures

were found to be similar to that reported by Corredor et al (2007) who reported 8% MC for bran from a dry decortication process.

Sample preparation was conducted to ensure the biomass was in the ideal state for compositional analyses investigations. This involved sieving the bran into fractions that were neither too fine nor too coarse. The recommended size for biomass analysis is -20/+80 which refers to the fraction that passes through the 20 mesh sieve but is retained by the smaller 80 mesh sieve (Sluiter et al, 2010). About 83.8% of the white bran and 87.1% of the red bran fell into this range. However, it was observed that the -80 fraction formed a considerable proportion of the brans, about 12-15%, and discarding it could use an overall change in composition as certain anatomical fractions segregate disproportionately into the fines fraction (Sluiter *et al.*, 2010). On the other hand, the amount of the +20 fraction was insignificant in both brans so that was also retained. In subsequent experiments, the brans were thus not sieved after milling.

Ash content represents the mineral/inorganic matter of the biomass removed after all the vapourisable, organic matter has been removed by combustion (Tiwari and Singh, 2012). The white and red brans had ash contents of approximately 1.8% and 1.5%, respectively. These figures are similar to a reported figure of 1.82% (dry weight basis) for sorghum grains (FAO, 1995). For comparison, these values are less than the ash content of herbaceous feedstock such as barley straw, alfalfa stems and canary grass reported to be 4.5%, 7.1% and 8.0% respectively (Amarasekara, 2013).

The carbohydrate content, including both storage and structural carbohydrates, was also determined as this was vital to give an indication of the carbon source for consideration for potential fermentative processes. The total content was determined by acid hydrolysis and it was observed that WB and RB had almost equal amounts of about 69.03 % and 70.48% respectively (**Table 3.4**); this is only slightly lower than 73 % reported for the whole grain (Fuleky, 2009). Glucose content was the highest at about 58 – 60 % and trace amounts of xylose, arabinose, mannose; cellobiose and maltose were not detected indicating that the digestion of cellulose and starch had proceeded to completion. These observed high carbohydrate values was a very significant finding as they indicate an abundance of potential carbon source in sorghum brans for bioconversion processes. It also indicates that the processing method is not very efficient resulting in high residual starch content in the bran.

Lignin is an amorphous polyphenolic compound formed from the polymerisation of three phenylpropanoid monomers namely coniferyl, sinapyl and *p*-coumaryl alcohols. Lignin has been shown to exist in cereal grains using the acetyl bromide (and other methods), even after excluding the possibility of interference by low molecular weight phenolic compounds (Bunzel *et al.*, 2004). The lignin contents of the brans were determined by the acetyl bromide method which is based on the solubilisation of lignin and the determination of absorbance values at 280 nm. This method is simple and quick to perform, and reportedly provides higher lignin recovery (Moreira-Vilar *et al.*, 2014). It was estimated that the lignin contents of WB and RB were 6.7 % and 10.5 % respectively. These **Figures** are higher than those reported for whole grains because lignin (along with cellulose and hemicellulose) is a structural material found in the pericarp, testa and aleurone tissues (see **Figure 1.9**) and mainly retained in the bran (Arendt and Zannini, 2013). They are however in congruence with the sum total of the insoluble dietary fibre and alkali-extracted dietary fibre for rye bran as reported by Bunzel *et al.* (2004).

In addition to carbon sources, microorganisms require other nutrients for growth and bioconversion processes. Protein is the second major component of sorghum (FAO, 1995), with the protein content indicating the presence of constituent amino acids which fermenting organisms require in order to proliferate. The red bran had a higher protein content at 16.9 % than white bran at 15.6 % protein, which is higher than the amount found in whole sorghum grains where a range of 8.6 % – 18.2 % has been reported (Virupaksha and Sastry, 1968). The sorghum grain contains up to 80 % of its protein in the endosperm (**Figure 1.9**) (Taylor and Schüssler, 1986), where they exist as protein bodies composed mainly of prolamin and in the protein matrix located in the peripheral and inner endosperm comprising mainly glutelin (FAO, 1995). However, the germ comprises albumins and globulins which form about 16 % of grain Nitrogen (Taylor and Schüssler, 1986) while 3% is located in the pericarp. The high bran protein content could be because the bran contains all of the pericarp content, in addition to parts of the endosperm and germ. This high protein content also indicates that brans may have a good potential for nutritional applications.

The crude lipid content of WB and RB were 2.3 % and 3.7 %, respectively which is quite similar to reported values of 2 % to 3 % for decorticated sorghum bran (FAO, 1995) and to that of whole sorghum reported to be 3.0 % (Corredor *et al.*, 2007) and 3.3 % by Jambunathan and Subramanian (1988). The similarity with whole grain values could be ascribed to the fact that the germ fraction which contains most of the oils has been ground

and mixed in the slurry from which the bran was extracted. The oils are thus uniformly dispersed by the milling process.

The summative mass closure of the sorghum brans were achieved with white bran being elucidated up to 102.9 % and RB to 109.4 %. The values surpassing the 100 % mark could be due to several reasons, such as an over estimation of the protein content due to the method used, and the conversion factor of 6.25 which is not specific for sorghum bran, or simply due to experimental error.

### 3.4.2 Other Compositional Analyses

Other parameters were determined to give a clearer picture of the composition of the sorghum brans. Thus, even though the total carbohydrate content had been determined, starch was specifically evaluated. From **Table 4.9** it was observed that both brans had a high starch content with 49.7 % and 53.0 % of the WB and RB respectively being composed of starch left after the slurry was filtered (see **Section 2.2.1**). This was a surprisingly high value considering that whole sorghum grains contain 56 % - 76 % starch (Jambunathan and Subramanian, 1988) and the sieving process was aimed at ensuring starch removal. However, high values have been previously reported with 16.4 - 33.2 %, reported for wheat bran, 51.0 % for barley bran and 52.3 % for oats bran respectively (Clegg, 1956; Englyst *et al.*, 1983; Bhatt, 1993). These high amounts observed in this work could be ascribed to the poor grinding ability of the machine used, and to inadequate squeezing and rinsing during the sieving process. It is however important to note that as the major component of sorghum, the variability of this method means sorghum bran residual starch content will vary within batches. These starch values contribute 82 % and 87 % of total glucose obtained from carbohydrate hydrolysis respectively with structural carbohydrate components being much lower at 5.7 % - 7.5 % and 5.2 % - 5.4 % for cellulose and hemicellulose respectively (**Table 3.10**). The cellulose and hemicellulose contents found in the present study were considerably lower than the 18 % and 11 % respectively reported by Corredor *et al.* (2007) but this could be due to the higher starch content observed in this work, and the fact that their process didn't involve milling the whole grain, but abrasive decortication which selectively removes the bran only. The discovery of such high amounts of starch was another very significant finding, as this indicated that starch degradation would be a suitable way to generate glucose-rich hydrolysates for subsequent fermentations.

Similarly, the reducing sugars were investigated by the DNS method as there was very little information available concerning this component of sorghum bran and its potential contribution to the bioconversion is worth noting. The values of 0.85 % and 1.16 % observed for WB and RB respectively (**Table 3.11**) were found to be higher than an average of 0.12 % reported by Jambunathan and Subramanian (1988) for whole grains. This might be due to the liberation of sugars from the starch by microbial action during the steeping/soaking process of bran preparation.

### **3.4.3 Hydrolysate Generation**

Having elucidated the composition of the brans, hydrolysates were prepared following two distinct methods: dilute acid and enzymatic starch hydrolyses.

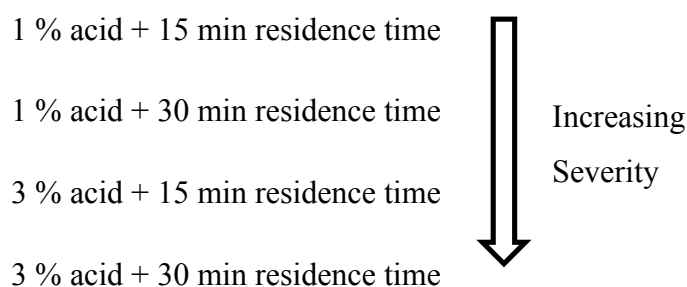
#### **3.4.3.1 Enzyme Hydrolysates**

In the enzymatic method a porcine pancreatic amylase and fungal amyloglucosidase were utilised. It was observed that the reaction proceeded rapidly in the first few hours and glucose production peaked about four hours into the incubation (**Figure 3.4**). The digestion was left to proceed for 24 h with intermittent sampling and the maximum glucose produced was 55 g/L and 61 g/L in WB and RB respectively. The higher value obtained from RB was expected as the RB had a higher starch content (**Table 3.9**). These values correspond to 49.9% of the theoretical maximum yield for white bran and 57.6 % for red bran, and are lower than the near 100 % conversion which is achievable by enzymatic hydrolysis and the 85 % reported by Corredor *et al.* (2007) in previous work with sorghum bran. The reasons for the incomplete digestion and these low glucose yields are not clear, particularly as DMSO was added during the hydrolysis to ensure the breakdown of resistant starch. The adequacy of the glucose concentration of the hydrolysate will be determined upon downstream utilisation.

#### **3.4.3.2 Dilute Acid Hydrolysate**

With dilute acid hydrolysis, the acid converts the starch into glucose alongside products of incomplete digestion such as maltose and oligosaccharides. Firstly, because there are various possible combinations of the acid and reaction conditions, an optimisation process was carried out using either low or high levels of the key parameters of residence time, acid

concentration and solid loading rate. The effects of dilute sulphuric and nitric acids at 1% w/w and 3 % w/w concentrations on bran at a 5 % solids loading rate were thus compared for bran conversion to simple sugars at 121°C at 15 min and 30 min residence times. It was assumed that the severity of the pretreatment conditions varied in the following format:



The impact of acid type was assumed to be similar and temperature taken into consideration as this was not varied.

The main sugars liberated in all conditions were glucose, maltose, xylose and arabinose (**Figure 3.5**) with trace amounts of galactose (not shown). It was observed that glucose was the most abundant sugar released with 51.5 % and 70.5 % of the bran being converted into this sugar at the lowest and highest severity conditions respectively using nitric acid and this was in line with expectation given that glucose is a product of the breakdown of starch (and to a lesser extent, cellulose) while the pentoses were from hemicellulose breakdown.

In the white bran digestion, it was observed that nitric acid was the slightly better performing acid, converting a maximum 70.5 % of the bran while sulphuric acid converted 69.1% of the bran sample (**Figures 4.3 and 4.4**), although t-test showed differences to be insignificant ( $t = 0.3647$ ;  $df = 1$ ;  $P = 0.7503$ ). Increasing the concentration of acid improved conversion as 3 % of both acids increased glucose release at both residence times; for instance  $\text{HNO}_3$  showed a 17.8 % increase in WB conversion to glucose from 1 % to 3 % while the increase was less pronounced with  $\text{H}_2\text{SO}_4$  at 10.2 %. Residence time had no effect on WB conversion to glucose at 1 % but increased output with both acids at 3 %, notably from 61.7 %- 70.5 % with nitric acid. This indicates that increased severity of high acid concentration at 121°C for a longer time improved bran degradation into glucose although the increase was not statistically significant ( $P = 0.06$ ). However, this can also result in the production of inhibitors, particularly at higher temperatures (Akanksha *et al.*, 2014). The impact of the various conditions on the other sugars was negligible.

With red bran, the trend was similar to that observed with white bran (**Figures 4.5 and 4.6**) whereby a higher concentration of acid resulted in higher bran conversion into simple sugars. Similar to the WB digestion, the choice of catalyst was important but here  $\text{H}_2\text{SO}_4$  was found to be better at digesting RB than  $\text{HNO}_3$ , producing a maximum of 66.8 % glucose compared to 60.6 % with  $\text{HNO}_3$ . Also, the effect of acid concentration was more pronounced in  $\text{H}_2\text{SO}_4$  than in  $\text{HNO}_3$  digestion of RB, with a 3 % concentration providing a 13.8 % and 6.7 % increase in RB conversion respectively. This was contrary to observations made by Akanksha *et al.* (2014) who found the lowest concentration of  $\text{H}_2\text{SO}_4$  optimal in digesting sorghum bagasse. However, their observation may be due to the fact that the digestion was performed at a higher temperature of  $150^\circ\text{C}$  which might considerably increase severity and hence efficiency of the pretreatment. Conversely, increasing the residence time had a negative effect on RB conversion at both concentrations of both acids with 3.5 % and 4.3 % decrease with  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$  respectively. The associated decreasing maltose contents with increasing severity did not result in increasing glucose liberation, which indicates that existing or newly generated glucose might be degraded into HMF. This is expected because high severity could produce/liberate inhibitory compounds during pretreatment while also reducing the amount of available sugars (Leenakul and Tippayawong, 2010; Akanksha *et al.*, 2014).

Overall, with regards to bran type, white bran was only slightly more amenable to conversion/digestion than red bran with the peak conversion being 70.5 % and 66.8 % respectively. As regards the residence time, increasing it resulted in an increased liberation of glucose in the WB especially with the nitric acid where an 8.8 % increase was observed; however it caused a decrease in glucose yields in RB with both acids and was the least beneficial parameter across all combinations. The reason for this decrease could be degradation of liberated glucose and xylose into HMF and furfural due to the longer residence time of 30 min. Acid type was also not very important, with only insignificant improvements in bran conversion by nitric acid over sulphuric acid which are outweighed by the higher costs of nitric acid over sulphuric acid. Acid concentration was however the most important parameter as up to 13% and 18 % increase in WB conversion to glucose was observed with increased sulphuric and nitric acid concentrations respectively (**Figure 4.4**). It was thus concluded that the ideal conditions for hydrolysate production was 3 %  $\text{H}_2\text{SO}_4$  for 15 min for both brans at  $121^\circ\text{C}$  and a pressure of 15 psi, using pressure-safe tubes in an autoclave.

#### 3.4.3.3 Solid Loading Ratio Optimisation

The optimum solids loading was investigated and it was observed that there was a higher bran conversion rate at lower SL. For instance, conversion into glucose and xylose were 23 % and 8 % at 20 % SL, compared to 41 % and 13 % at 10 % SL respectively. This trend is likely due to the fact that at low SL there was adequate contact between the acid and bran particles and digestion could proceed to completion while at higher SL, the bran particles settled at the bottom of reaction pressure tubes resulting in compaction and decreased digestion.

By contrast, at these same SL levels the actual concentration of glucose and xylose yields were higher at the higher SL levels; 47 g/L and 16 g/L respectively at 20 % WB SL rate, and 41 g/L and 13 g/L respectively at 10 % SL (**Figure 3.9**). This was consistent with the higher amounts of bran available at the higher solids loading. Assuming a unitary conversion rate, glucose and xylose released from the brans at 20% solids loading should be double that at 10 % SL and four times higher than that at 5 % SL, while the amount of sugars released in the hydrolysate (g/L) should decrease by half at each step decreasing from the 20 % rate. This is obviously not the case and both the SL and its interaction with RB significantly affected the amount of sugars released. A similar observation was made with RB where bran conversion efficiency dropped significantly compared to lower SL and the increase in glucose amounts from 42 g/L to 52 g/L with from 10 % SL to 20 % SL was not commensurate with the decline in efficiency of bran digestion from 44 % to 27 %. It has been reported elsewhere that solids concentration can determine subsequent concentrations of released sugars (Yang and Wyman, 2008).

It was thus concluded that even though the higher SL of 20 % produced more glucose and other fermentable sugars than either 5% or 10 % from both brans, the latter value will be used to produce the dilute acid hydrolysates. This is firstly because the increase in glucose liberation was more significant between 5 % and 10 % than between 10 % and 20 % SL (**Table 3.13**). In addition, there is considerable waste/loss of undigested biomass at the 20 % SL. This value is also similar to the 13.1 % reported for dilute acid pretreatment of agave bagasse (Ávila-Lara *et al.*, 2015).



### 3.4.4 Sample Hydrolysates

The sugar content of the hydrolysates varied from batch to batch, and this can be explained by variations in water loss during autoclaving. However, the amounts were within the same range as represented in **Table 3.14**. The RBDH was coloured bright red while the other hydrolysates were various shades of cream colour. The RBEH did not take up the colour of the bran because the enzyme only dissolves the starch and does not act on the phenolic flavanoid pigments located in the pericarp into solution.

### 3.4.5 Inhibitor Analysis

The inhibitors detected in the bran hydrolysates were generally in relatively low quantities, with the highest observed amount being that of acetic acid which ranged from 22 to 43 mg/l of hydrolysate (**Table 3.15**). While this could be produced during pretreatment explaining its presence in the DAHs, it is considered more likely that the presence in the enzyme hydrolysates was due to the acetate buffer employed in hydrolysate preparation.

The possible impact of each inhibitory compound varies with the inhibitor and with the strains employed in the bioconversion process. A concentration of 1 g/L of vanillin has been shown to cause a reduction of 25 % in ethanol yield in fermentations with *S. cerevisiae* while the oxidised form, vanillic acid showed no inhibition at concentrations up to 1 g/L (Ando *et al.*, 1986). Furfural is usually found in lower levels than HMF (Almeida *et al.*, 2007) but inhibits specific growth rates and ethanol productivity in ethanologenic yeast at levels of 1 g/L. It can also be converted by yeasts to furfuryl alcohol which can inhibit *S. stipitis* and to a lesser extent *S. cerevisiae* growth (Palmqvist and Hahn-Hagerdal, 2000). At 17 g/L furfural almost completely suppressed *S. cerevisiae* growth and all cells died after 8 h (Lin *et al.*, 2009). The amounts of furfural detected in the hydrolysates in the current work were considerably lower than this lethal dose at 0.33 mg/l to 5.95 mg/l and none were detected in the RBEH, thus indicating that the conditions of the hydrolysate preparation were sufficiently mild to avoid pentose degradation to furfural. Ferulic acid is inherent in some cereal grains and little information is available on its presence in hydrolysates. **Table 3.16** shows that the hydrolysates prepared from sorghum bran in the current study compare favourably to others reported from lignocellulosic biomass by various authors. It was therefore considered that the hydrolysates produced in the current work were relatively safe for fermentation purposes

given that the levels of potential inhibitory compounds were lower than those reported from the various hydrolysates presented in **Table 3.16**

**Table 3.16:** Concentrations of common inhibitory compounds present in various lignocellulosic hydrolysates

COMPOUND	CONCENTRATION (g/L)				REFERENCE
	Spruce	Corn Stover	Wheat	Sugar cane	
HMF	5.9	0.06	-	0.6	Almeida et al (2007)
Furfural	1.0	11	-	1.9	" "
Acetic acid	2.4	1.6	1.6	4.4	" "
Vanillic acid	0.034	-	0.067	-	" "
Ferulic acid	-	-	-	-	

### 3.5 Summary And Conclusions

- White and red sorghum brans were milled and their composition determined. The brans were found to contain about 50 % residual starch which was higher than expected. This residual high starch content was considered to be an adequate potential carbohydrate source for sugar generation. As the brans were also rich in protein, lipids and ash it was concluded that the feedstock showed potential for downstream bioprocessing.
- The brans were then subjected to two types of pretreatment processes, dilute acid and enzymatic pretreatments, to attempt to release sugars for later use in fermentations. Significantly different amounts of sugars were produced according to the conversion process and conditions used. A maximum of 70.5%, 6.2 % and 9.7 % of the WB was converted into glucose, xylose and arabinose, respectively, when utilising DAH. Acid type and retention time did not make a significant impact on the bran conversion efficiency, whereas an increase in concentration of acid significantly impacted sugar release. Meanwhile, enzymatic processing was concluded after 6 h and between 50 % and 58 % of the brans were enzymatically converted into glucose.
- Investigation of solid loading rates for the DAH revealed that more of the bran was digested at higher loading rates but not at a commensurate rate i.e. even though the amount of liberated sugars increased with SL rate, the efficiency of bran conversion

dropped which made the 20 % higher SL undesirable. Although the 5 % rate was most efficient in achieving bran conversion into sugars, the actual concentrations were considered low for downstream processing uses. Therefore a 10 % SL value was chosen as optimal, which allowed conversion of approximately 41 % and 12 % of the bran to glucose and xylose respectively.

- Sulphuric acid was chosen as the best pretreatment option for various reasons. The cost was considerably less than nitric acid with little difference in performance, and similarly the higher cost of the enzymes relative to the cost of the very dilute acids made enzyme hydrolysates less desirable.
- The optimum conditions for later work were therefore chosen to be digestion of a 10 % SL of either bran in 3 % sulphuric acid at 121°C and 15 psi for 15 min.
- The hydrolysates produced were also found to contain considerably less inhibitors than other biomass hydrolysates reported in literature, and the amounts detected were below lethal level for fermenting organisms. The hydrolysates thus appeared ideally suited for downstream biomass conversion processes.

## **Chapter 4: Production of Value-Added Chemicals from Sorghum Bran**

This chapter aims to determine the suitability of sorghum bran produced from the wet milling process as a feedstock for the fermentative production of ethanol and itaconic acid. Several fungal isolates/strains were investigated and screening experiments conducted. The most suitable strains were then used to ferment the hydrolysates to determine productivity and yields.

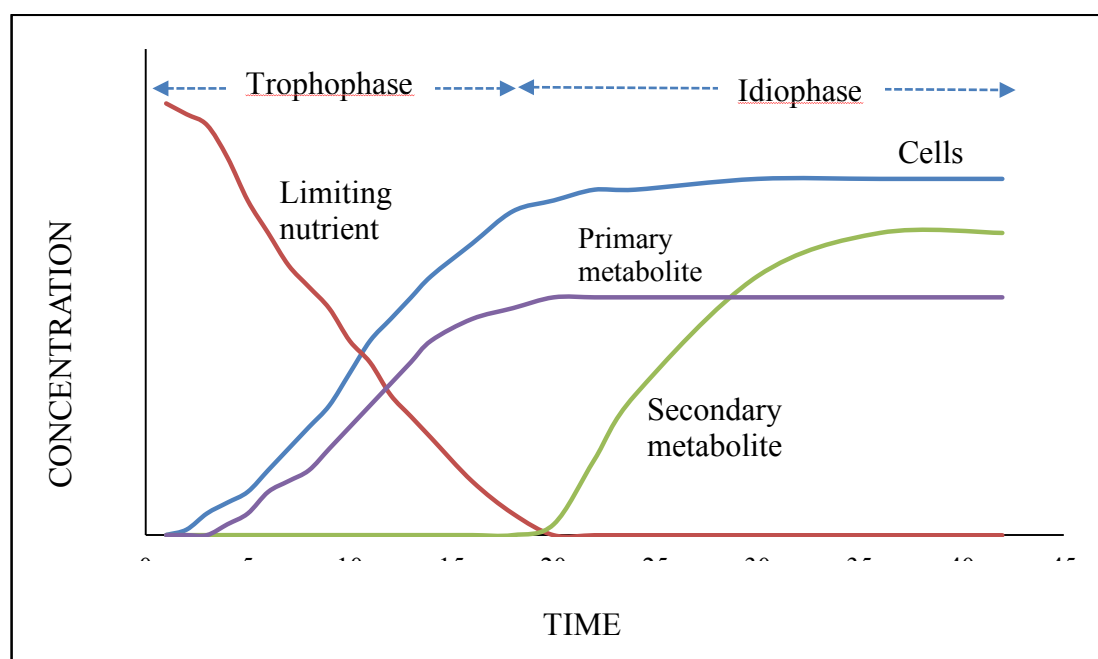
### **4.1: Introduction**

A natural product is a substance produced by a living organism and is found in nature even if it can be produced synthetically. Natural products can be classified based on their cost of production and amount produced annually. Low-value, high-volume products are those whose cost is low at around £6/kg but which are required in large quantities (millions of kg per year) such as citric acid. High-value, low-volume products are usually bioactive products which are required in only small amounts throughout the world annually (a few kg) but the price may be as high as £60/mg e.g. recombinant proteins and growth hormones (these figures cited are mainly indicative and not absolute). Medium value, medium-volume products fall between both extremes (Hunter, 2007). Depending on the stage of the organism's growth at which the product is produced, natural products can be divided into primary and secondary metabolites.

Primary metabolism involves processes that are crucial to the growth, development or reproduction of the organisms. Primary metabolites are required during the active growth stage and perform essential and obvious physiological functions and must be synthesised by the host organism if not present in the growth medium. They are associated with the rapid initial phase of fungal growth, and are thus found in a wide range of species. Examples include amino acids, vitamins and fermentation end products such as ethanol and organic acids (Horgan and Murphy, 2011).

The products of primary metabolism usually provide substrates for secondary metabolism. In contrast to primary metabolites, secondary metabolites are produced from secondary

metabolic processes which are not essential for the growth or survival of the organism and are produced within a more restricted taxonomic range of organisms (Demain, 2005). They are also called idiolites as they are usually not produced in the trophophase (log/exponential phase), but in the idiophase (stationary/production phase) after cell division has stopped due to exhaustion of key nutrients or oxygen, or pH changes have set in (**Figure 4.1**). Common secondary metabolites include organic acids, antibiotics etc. Fungi, particularly members of genus *Aspergillus* frequently over-produce organic acids which provides an ecological advantage as many species thrive below pH 4.0 while rapidly growing bacteria and some fungi cannot grow below pH 3.0 (Magnuson and Lasure, 2004; Kubicek *et al.*, 2010).



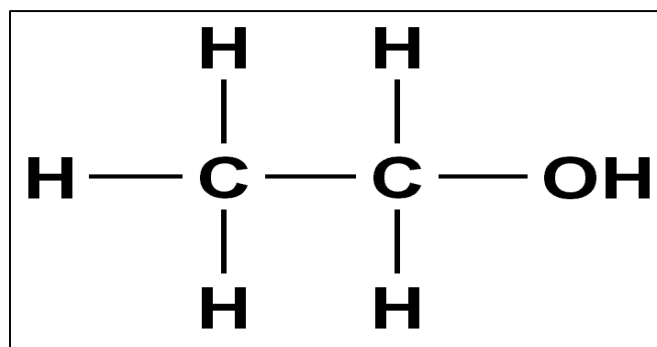
**Figure 4.1:** Primary and secondary metabolite production in microorganisms

While secondary metabolites can serve as sex hormones or competition strategies against other organisms etc., in many cases, their exact function in the cell is not known (Nielsen *et al.*, 2012). However, they can have profound biological properties of relevance to mankind and this has been extensively commercially exploited (Horgan and Murphy, 2011). Other secondary metabolites include pigments such as astaxanthin produced from *Phaffia rhodozyma*, mycotoxins such as fumonisins produced by *Fusarium* species, and pesticides etc.

The fermentative production of one primary metabolite ethanol and one secondary metabolite itaconic acid from sorghum bran will be investigated in this chapter.

#### 4.1.1 Ethanol

Ethanol ( $C_2H_5OH$ ) is the second member of the aliphatic alcohol series. Ethanol is also known as ethyl hydrate, ethyl alcohol or informally as alcohol (**Figure 4.2**). It is a volatile liquid with many uses including as a psychotropic drug, a solvent, and as a fuel among other industrial and domestic applications. It is a psychoactive drug consumed recreationally and is the primary alcohol found in alcoholic beverages (O'Leary, 2000).



**Figure 4.2:** Structure of ethanol ([www.commonswikimedia.org](http://www.commonswikimedia.org) )

Renewable ethanol is generally considered to be an environmentally beneficial energy source because its emissions on combustion are less toxic than those of petrol; its production recycles the  $CO_2$  produced from its combustion and because it reduces dependence on fossil fuel (Balat, 2011). It was first used in 1897 in the internal combustion engine by Nikolas Otto (Rothman *et al.*, 1983) and can be used in various blends in engines that use petrol (Balat *et al.*, 2008). If its production process is powered with renewable energy, then there is no net addition of  $CO_2$  to the atmosphere. With the inevitable depletion of petroleum resources and the drive for sustainability discussed in **Section 1.1.3**, coupled with its ease of manufacture, renewable ethanol has been increasingly favoured and is currently considered the cleanest liquid fuel alternative (Lin and Tanaka, 2006). It is now the most widely used biofuel in transportation globally (Balat, 2011).

#### **4.1.1.1 Physical Properties of Ethanol**

Ethanol is a volatile colourless liquid which is completely miscible in water and organic solvents and is very hygroscopic. It has a refractive index of 1.36, a pleasant odour and a burning taste. Ethanol has a density of 0.79 g/cm<sup>3</sup>, a melting point of -114°C and boiling point of about 78.4°C. It is very flammable and burns with a smoke-less blue flame. It is commonly available as a 95% azeotrope but can also be obtained as absolute (100%) ethanol (O'Leary, 2000).

#### **4.1.1.2 Chemical Properties of Ethanol**

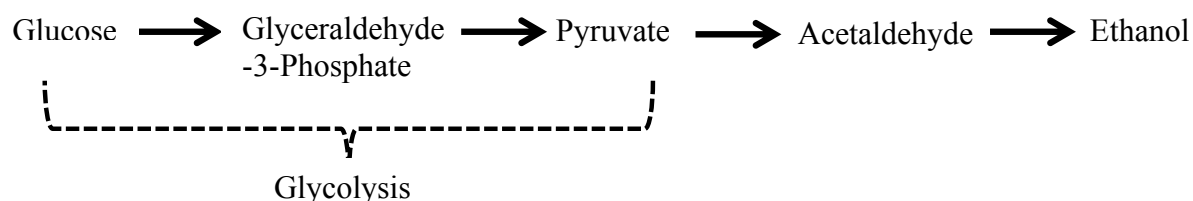
Ethanol has a molecular weight of 46 g/mol. It has about 2/3 the energy density of petrol but a higher octane rating (a measure of fuel quality), leading to improved efficiency and performance. Since it is an oxygenated fuel (35% oxygen) it burns with fewer particulates and nitrous oxide emissions upon combustion (Balat *et al.*, 2008).

Ethanol participates in various chemical reactions including oxidation to ethanal and then ethanoic acid; dehydration to ethane; esterification with organic acids and halogenation among several others (O'Leary, 2000).

#### **4.1.1.3 Ethanol Biosynthesis**

Ethanol fermentation is a biological process that involves the decomposition of one mole of glucose into two moles of product. Ethanol is produced by several microorganisms referred to as ethanologens which are mainly yeasts such as *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, *Kluyveromyces marxianus*, *Schizosaccharomyces pombe* and *Candida shehatae*. It converts hexoses such as glucose and fructose to pyruvate via glycolysis which is then eventually converted into ethanol (Cardona *et al.*, 2009; Greetham *et al.*, 2014).

A simplified illustration of ethanol biosynthesis pathway in *S. cerevisiae* is summarised in equation below.

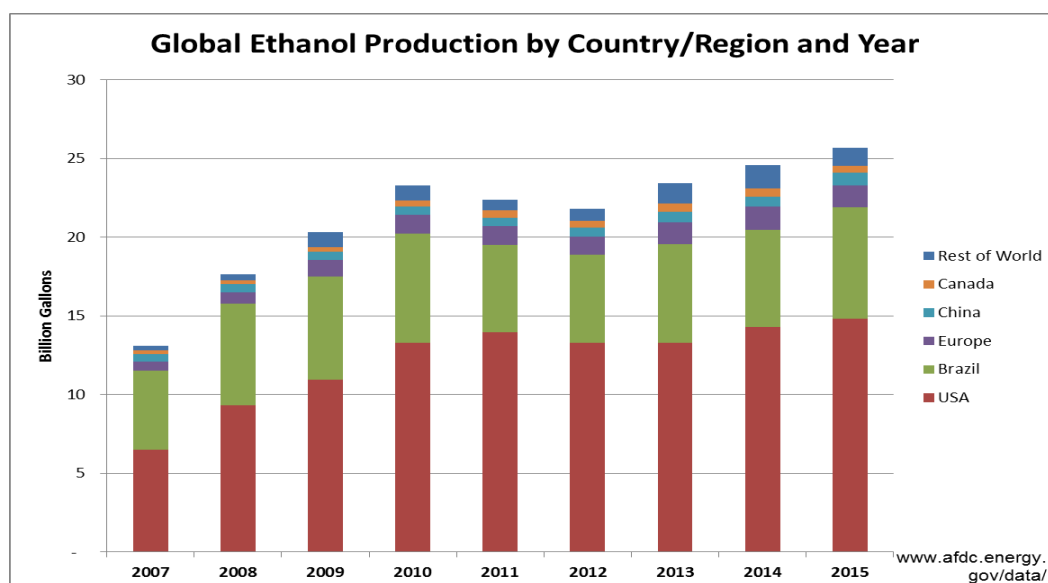


*Zymomonas mobilis* rapidly ferments glucose into ethanol via the Entner-Duodoroff pathway (Macedo and Brigham, 2014).

#### **4.1.1.4 Commercial Ethanol Production**

Ethanol is the oldest synthetic organic chemical produced by man (Gupta and Demirbas, 2010). It is produced by two major industrial routes. Ethanol required for industrial use is prepared by the hydration of steam, whilst that required for food and fuel uses is mainly produced by fermentation (O'Leary, 2000). Recently ethanol production has been increasing annually and has reached the highest recorded levels in the United States. The Global Renewable Fuels alliance (GRFA) an international federation representing most of the world's renewable fuels production reported that 88 billion litres of bioethanol were produced in 2013 (Baker, 2014). Brazil and the USA produce over 80% of the world's ethanol annually (Demirbas, 2009) by the fermentation process (**Figure 4.3**).





**Figure 4.3:** World leading producers of bioethanol (AFDC, 2016)

Brazil has conducted a bioethanol program since 1931. By 2011 it produced over 62,000 m<sup>3</sup> of ethanol from sugarcane daily and in 2012 it set a rate of 20-25% anhydrous ethanol to gasoline blend (Koizumi, 2014). The USA produces over 144,000 m<sup>3</sup> daily (Kamm, 2014; Koizumi, 2014) mainly from maize.

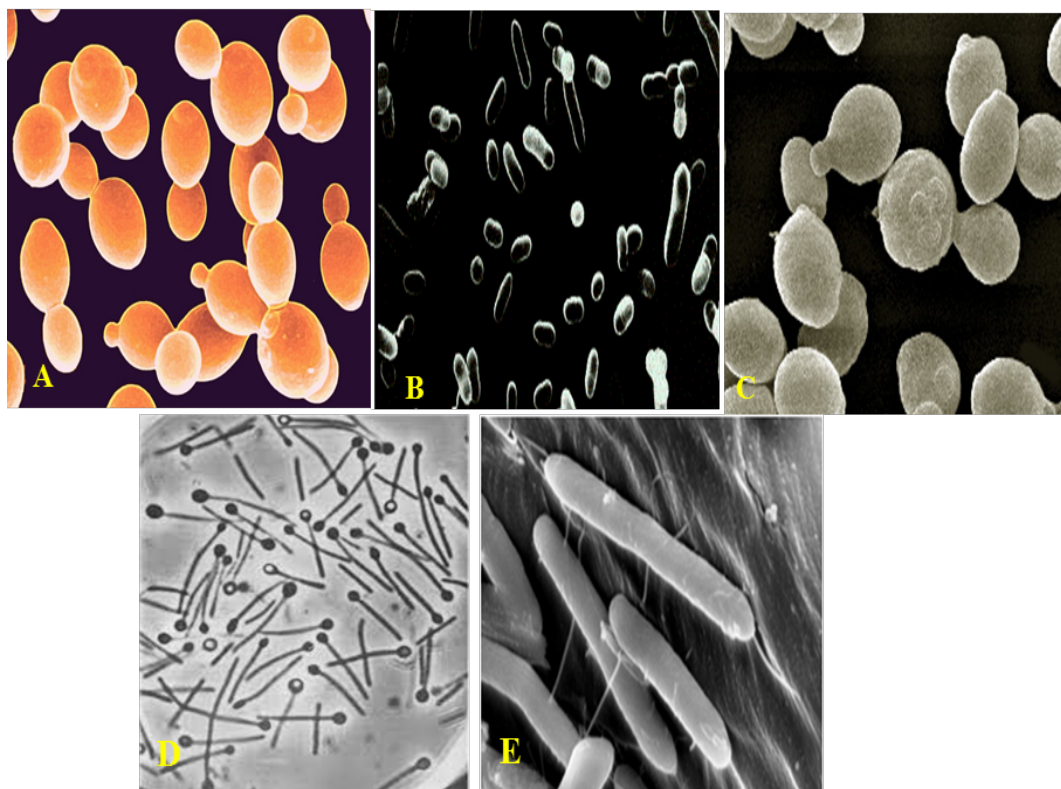
Several countries have enacted policies to support and increase the uptake of biofuels in transport. Renewable chemicals are also being supported with improved access to capital and improving legislative support in the USA (Young and Jalbert, 2016).

## Microorganisms

Yeasts have several advantages for the industrial production of ethanol, for instance they are able to tolerate high concentrations of up to 150 g/l of ethanol in the fermentation broth before product inhibition sets in (Cardona *et al.*, 2009). Also, *S. pombe* is able to tolerate high concentrations of salts and solids in the medium while *K. marxianus* can grow under thermophilic conditions which make them ideal candidates for simultaneous cellulose hydrolysis and fermentation as cellulases function at higher temperatures (Ballesteros *et al.*, 2004). The *S. cerevisiae* yeast strains are the most widely employed ethanologen. They are capable of converting hexose sugars to carbon dioxide under aerobic conditions to produce mainly biomass as in the production of baker's yeast, but produce mainly ethanol under anaerobic conditions (Cardona *et al.*, 2009).

A key limitation of *S. cerevisiae* is that it is unable to assimilate the pentoses liberated from the hydrolysis of hemicellulose such as xylose and to a lesser extent, arabinose. Consequently, several yeasts which can assimilate both hexoses and pentoses such as *S. stipitis* and *C. shehatae* are being utilised. However, these organisms will also more readily utilise and exhaust the hexoses, then switch to the pentoses after a short lag phase. The ethanol productivity and tolerance are however considerably lower than in *S. cerevisiae* and their cultures require oxygenation.

Bacterial ethanologens have also been investigated and these include the thermophiles *Clostridium thermohydrosulfuricum*, *C. thermosaccharolyticum* and *C. thermocellum* (**Figure 4.4**). These organisms have several advantages including the ability to transform pentoses into ethanol at high temperatures; saccharolytic properties which make them capable of converting untreated wastes such as lignocellulose into ethanol (Macmillan, 1997 in Cardona et al., 2009).



**Figure 4.4:** Various ethanol producing microorganisms. A: *S. cerevisiae*; B: *Z. mobilis*; C: *S. stipitis*; D: *C. thermosaccharolyticum* E: *C. thermocellum*

(Image credits A: [www.bbsrc.ac.uk](http://www.bbsrc.ac.uk) B: [www.ejbiotechnology.info](http://www.ejbiotechnology.info) C: [genome.jgi.doe.gov](http://genome.jgi.doe.gov); D: [www.aem.asm.org](http://www.aem.asm.org); E: [www.lookfordiagnosis.com](http://www.lookfordiagnosis.com))

The main limitation of these organisms is their low ethanol tolerance of under 30 g/l; in addition, they produce several by-products and thus have low ethanol yields. However, the bacterium *Zymomonas mobilis*, like *S. cerevisiae*, produces ethanol in quantities approaching the theoretical maximum in its native form (Darku and Richard, 2011) and both organisms are homo-ethanol fermentative. *Z. mobilis* is a facultatively anaerobic, Gram-negative bacterium which can utilise both hexoses and pentoses under anaerobic conditions, has a high tolerance of over 100 g/l, requires no addition of oxygen, is quite amenable to genetic manipulations, and ferments at a higher temperature. Its drawbacks include that it can only utilise a narrow range of substrates limited to simple sugars such as glucose, fructose and sucrose; the formation of levan which increases viscosity during fermentation of sugarcane syrup; and the production of sorbitol which decreases conversion efficiency (Cardona *et al.*, 2009; Macedo and Brigham, 2014).

Genetic modification techniques are now being used to try and optimize the fermentative abilities of various bacterial and yeasts, with the greatest successes being seen in the modification of Gram-negative bacteria. One method involved the integration of pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase genes II (*adh II*) from *Z. mobilis* into *Escherichia coli* followed by introduction of the fumarate reductase gene to enable it produce up to 95% of theoretical maximum yields under anaerobic conditions. Similarly, the integration of the PET (Production of Ethanol) operon (comprising *pdc* and *adh II* co-expressed under the native *lac* promoter) into *E. coli* resulted in high productivities of up to 0.72 g/l/h and hyper-tolerance to ethanol inhibition (Dien *et al.*, 2003). *S. cerevisiae* has also been modified to be able to utilise xylose by the introduction of xylose isomerase genes into its genome (Macedo and Brigham, 2014).

## **Feedstock**

Globally, the production of ethanol is carried out by the fermentation of raw materials rich in carbohydrates. There are three major classes of these feedstocks namely sugar-based feedstock such as sugar cane, beet, sweet sorghum; starchy feedstocks including maize, sorghum, cassava and wheat; and lignocellulosic feedstocks such as straw and wood.

Sugar-based materials have a unique advantage over other feedstock in their simplicity, they do not require a prior hydrolysis step as sucrose can be broken down and utilised by yeasts. Sugarcane is the main feedstock used in global ethanol production. It is more widely

employed in tropical countries. For example, about 79% of Brazil's ethanol is produced from fresh sugarcane juice while sugarcane molasses accounts for the rest and for the bulk of India's ethanol production (Cardona *et al.*, 2009). Beet sugar is also used in ethanol production in temperate regions of the European Union such as in France which is Europe's largest bioethanol producer; and in the United States. Sweet sorghum is similar to sugar cane in that it contains a sugar-rich juice in its stalk and is also used in tropical regions. Sweet sorghum also has the additional benefit of drought resistance (Demirbas, 2009). The main draw-back of sugar-based bioethanol substrates is the fact that they compete directly with the food uses of these crops; and their perishability.

Starchy feedstocks include grains such as maize which is used to make up to 90% of all the bioethanol in the United States (Balat *et al.*, 2008), and wheat which is widespread in Europe. In addition to the juice, sweet sorghum also contains a grain head which is starch-rich and which is usually hydrolysed and fermented too, or used in the production of animal feed. The starch is hydrolysed into glucose syrup which is then fermented into ethanol. Amylases and amyloglucosidases are usually used to enzymatically saccharify the starch to sugars (liquefaction) prior to fermentation by microorganisms (Demirbas, 2009). Alternatively, dilute acid could also be used to achieve the hydrolysis of the starch polymer.

Maize ethanol production in the U.S. causes more soil erosion and uses more nitrogen fertilizer than any other crop grown; as is the case with sugar cane production in Brazil (Pimentel, 2003). Furthermore, starch-based bioethanol production is expensive because of the cost of cooking the starchy material before liquefaction (Balat *et al.*, 2008) and the high cost of liquefying and saccharifying enzymes, prior to fermentation. In addition, this 1<sup>st</sup> gen process competes with food production and negatively impacts food prices, as a result of which the process is also discouraged as elucidated in Sections 1.3 and 1.4 and is being phased out in favour of 2<sup>nd</sup> gen processes.

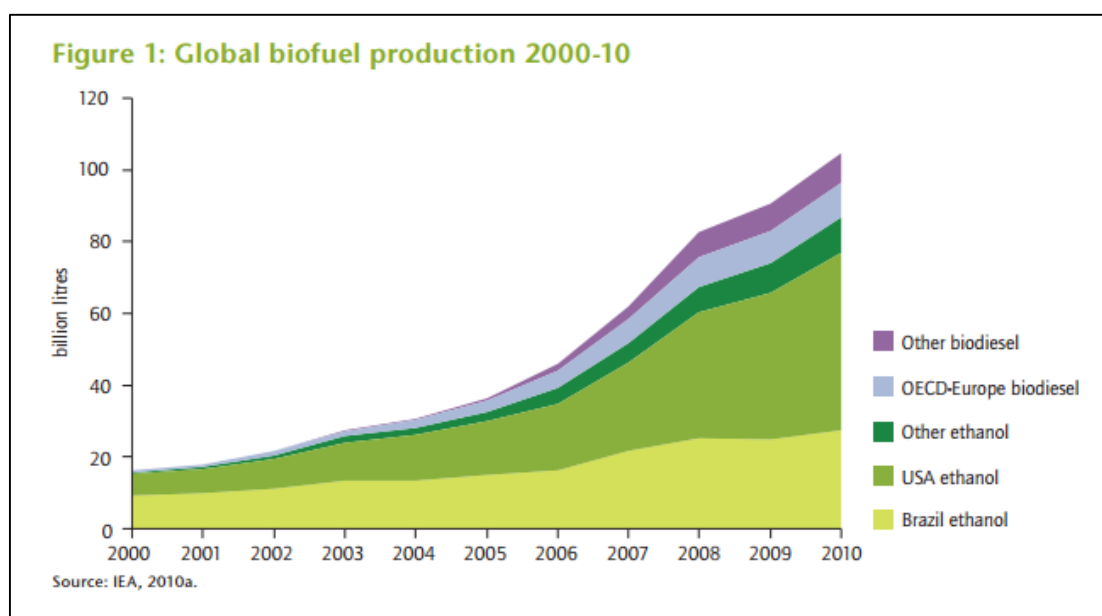
Second generation bioethanol addresses the challenges of the use of food sources. The use of two lignocellulosic materials, agricultural residues and wasted crops, could potentially yield up to 491 billion litres of bioethanol per year (Kim and Dale, 2004). However, the use of lignocellulosic feedstock is still very limited because of the aggressive and expensive pretreatment processes required to overcome the recalcitrance of lignocellulose and the low yields obtained (Gupta and Demirbas, 2010).

#### 4.1.1.5 Industrial Applications of Ethanol

Ethanol is a versatile organic chemical and it is an integral part of humans' daily lives. It is employed in a wide range of uses including domestic and industrial applications.

### Transportation Fuels

As discussed in preceding sections, the most important industrial/commercial application of ethanol is as a transportation fuel. Ethanol was by far the most widely used transportation biofuel in the last decade (**Figure 4.5**), and the IEA estimates that by 2030 biofuels use will have increased but that bioethanol will remain the dominant biofuel (Eisentraut, 2011).



**Figure 4.5:** Global biofuel production 2000-2010 (Eisentraut, 2011).

Most of Brazil's bioethanol is used domestically to substitute 40% of local petrol consumption while another 20% is exported (Balat *et al.*, 2008). Ethanol is used in combinations with petrol known as "blends". The most common blend in the USA is the 10% bioethanol to 90% petrol blend known as E10 or "gasohol"; while gasohol in Brazil is 24% bioethanol to 76% petrol (Balat *et al.*, 2008). Higher blends above E10 such as the E85 usually require engine modifications or are used exclusively in Flex-Fuel Vehicles (FFVs),

cars with internal combustion engines designed to run on more than one fuel. Ethanol is also used in bipropellant rockets.

### **Alcoholic Beverages**

The most common nonfuel use of ethanol is in alcoholic beverages such as wines, spirits and beers. It is the only alcohol that can be safely drunk in moderate quantities (Ramsden, 2001).

### **Solvent**

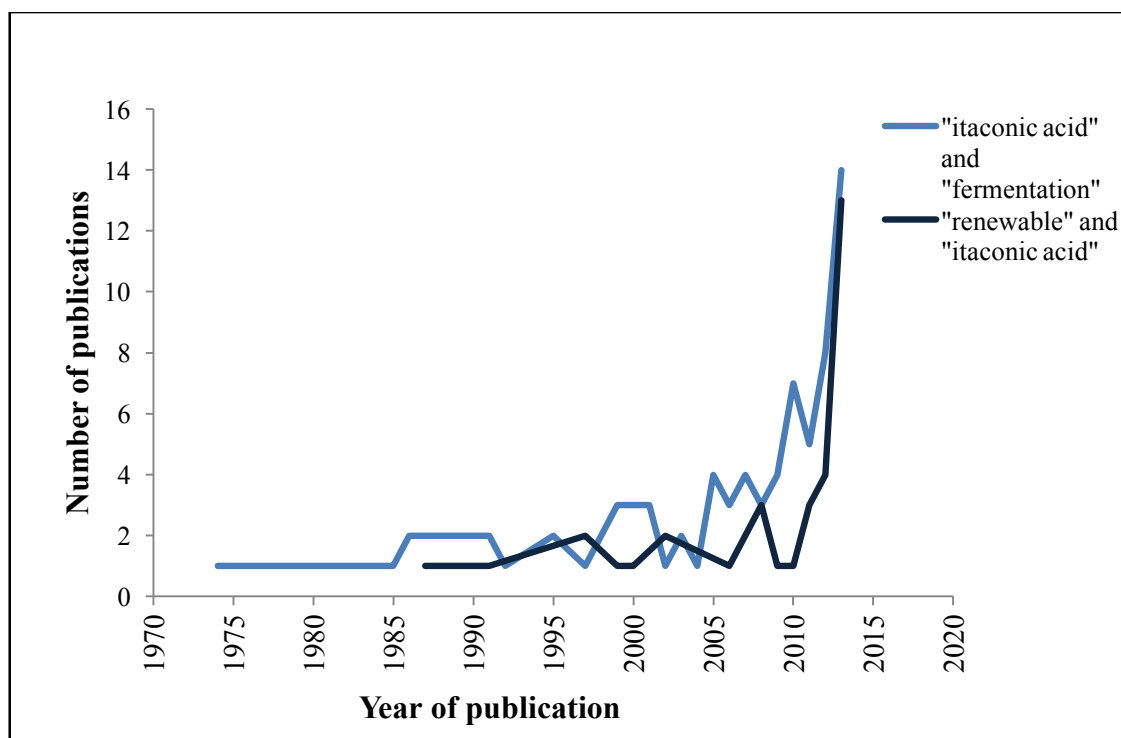
Ethanol is the second most widely used solvent in industrial and consumer products after water as it can dissolve a range of both polar and non-polar substances. It is a widely used solvent for paints, varnishes, cosmetics, toiletries and drugs (O'Leary, 2000; Ramsden, 2001) and being volatile, it evaporates and easily, leaving the solute behind.

#### **4.1.2 Itaconic Acid**

Itaconic acid (IA) is an organic acid that was first described by Baup who found it in 1837 as a product of citric acid distillation (Klement and Büchs, 2013). It is also known as methylene succinic acid or methylene butanedioic acid. It was discovered by Kinoshita in 1931 as a metabolite of a strain of *Aspergillus* which was subsequently named *Aspergillus itaconicus*. It was shown in 1939 by Calam and other workers that *A. terreus* can also accumulate IA in even greater amounts than *A. itaconicus* (Klement and Büchs, 2013).

The Northern Regional Research Laboratory (NRRL) of the United States Department of Agriculture (USDA) in Illinois screened several wild-type strains and identified *A. terreus* NRRL 1960, an isolate from Texas soil, as the most prolific itaconic acid-producing strain (Lockwood and Reeves, 1945). This then went on to become the most widely studied strain, being utilized by several other authors (Kautola *et al.*, 1985; Gyamerah, 1995b; Gyamerah, 1995a). At the same institute, preliminary attempts were also made to develop a biotechnical process for itaconic acid production, followed by optimisation of the industrial processes, in order to provide a marketable source of itaconic acid. The main developments in IA production (batch fermentation, free suspended biomass) took place before about 1966. Over

the next 15 years, the interest in IA production declined, as indicated by the few publications during this time (**Figure 4.6**).



**Figure 4.6:** Trend concerning itaconic acid research publications on Web of Science (Ahmed El-Imam and Du, 2014)

As discussed in **Section 2.5**, there have however been increasing general concerns regarding sustainability, environmental conservation, renewable resources and rising energy costs. This has resulted in the development of new fermentation technologies such as novel fed-batch strategies and continuous processes using immobilised cells (Willke and Vorlop, 2001). Together with the development of more sophisticated bioprocess control, this has jointly led to renewed interest in improving IA production (**Figure 4.6**).

#### 4.1.2.1 Physical Properties of Itaconic Acid

IA exists as white to light beige crystals (**Figure 4.7**) which dissolve in water up to 80.1 g/l at 20°C which makes it quite easy to purify by crystallization (Milsom and Meers, 1985). It has a density of 1.573 g/ml at 25 °C, a melting point of 165-168 °C and a flash point of 268°C (Pomogailo *et al.*, 2010).

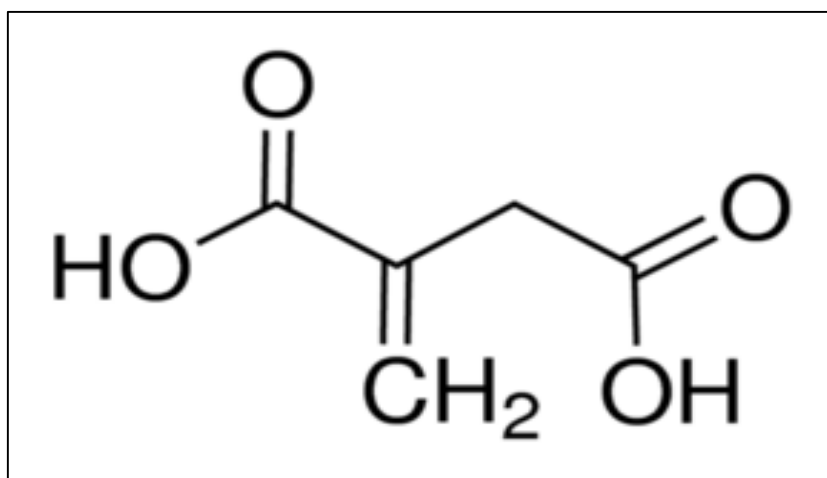


**Figure 4.7:** Itaconic acid crystals

Itaconic acid dissolves well in several alcohols such as methanol, 2-propanol, and ethanol with the solubility increasing at higher temperatures (Yang *et al.*, 2012). It is conveniently separated using ion exchange chromatography and detected by UV-spectroscopy at a wavelength of 210 nm (Welter, 2000) or using refractive index (RI) detectors.

#### **4.1.2.2 Chemical Properties of Itaconic Acid**

Itaconic acid is an unsaturated 5-C carboxylic acid (**Figure 4.8**) with the stoichiometric formula  $C_5H_6O_4$  and a molar weight of 130.1 g/mol.



**Figure 4.8:** Structure of itaconic acid ([www.sigmaaldrich.com](http://www.sigmaaldrich.com))



It has three protonation states with pKa values ranging from 3.83 - 5.55. With a degree of reduction of 3.6, it is just a little more oxidised than glucose with a value of 4.0 (Klement and Büchs, 2013). It is not used in food applications because it is slightly toxic, a mild irritant in humans and may inhibit succinic acid utilization in rats fed large doses (Clayton and Clayton, 1994). However, it is invaluable as a monomer because of its unique chemical properties, deriving primarily from its methylene group (**Figure 4.8**) which makes it able to participate in polymerisation reactions. In addition as a result of its possession of two carboxylic acid groups, IA heteropolymers have hydrophilic properties (Ruijter *et al.*, 2002). Itaconic acid can either be self-polymerised to polyitaconic acid (PIA) or can act as a co-monomer with other different monomers such as acrylamide (Willke and Vorlop, 2001).

#### **4.1.2.3 Itaconic Acid Derivatives**

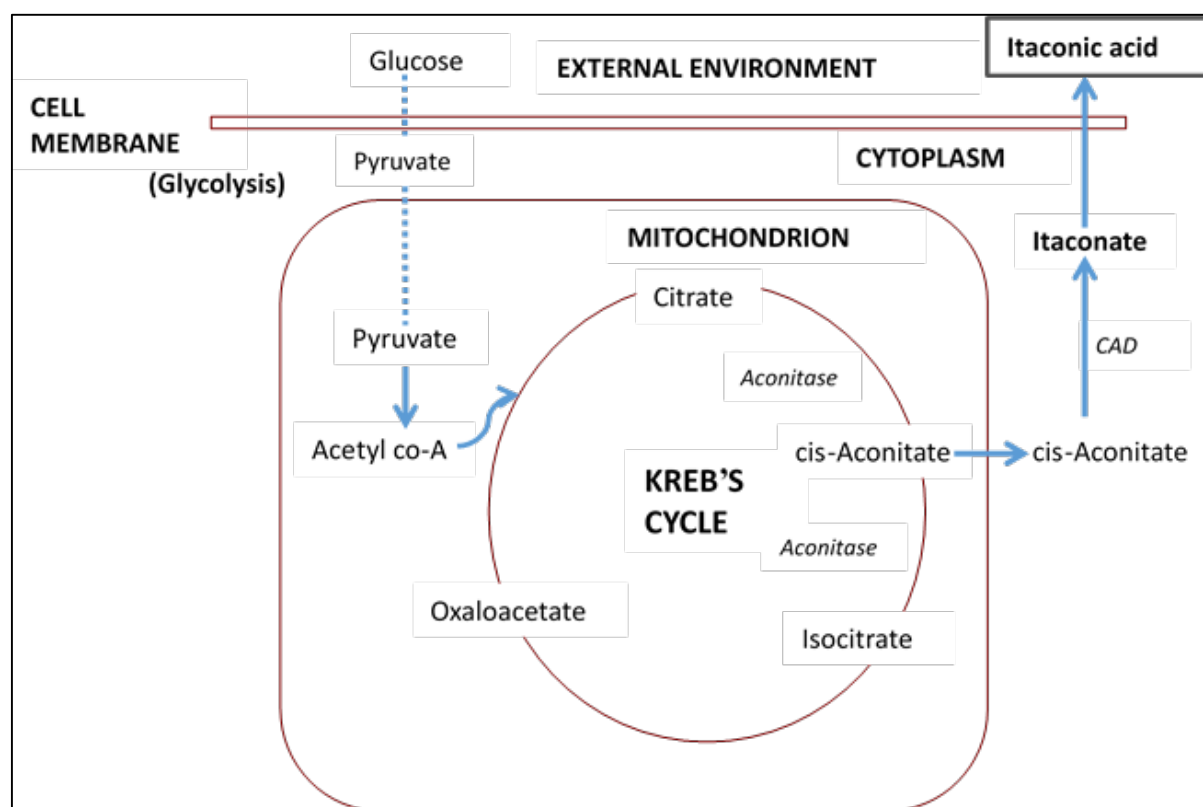
Itaconic acid forms a range of metallic salts. The calcium salt is more soluble than calcium citrate and thus cannot be used as readily as the latter in purification by precipitation. IA also readily forms diesters such as dimethyl itaconate and di-*n*-butyl itaconate both of which are readily available commercially. Itaconic anhydride may be used for the preparation of mono esters such as monomethyl itaconate. Itaconic acid also partakes in a reaction with amines to yield *N*-substituted derivatives of  $\alpha$ -pyrrolidones with actual or proposed uses in greases, detergents, shampoos, herbicides and pharmaceuticals (Wabnitz *et al.*, 2014). A condensate of lauric acid and aminoethylethanolamine reacts with IA to give an imidazoline derivative which is an active ingredient in shampoos (Milsom and Meers, 1985).

#### **4.1.2.4 Itaconic Acid Biosynthesis**

The process of the biosynthesis of itaconic acid in *Aspergillus terreus* has been a subject of academic discourse and has only recently being agreed upon unlike, for example, that of citric acid which has been well-established for decades. In 1931, when the production of itaconic acid was first discovered by Kinoshita in *Aspergillus itaconicus* (which in reality is likely to be an *Aspergillus terreus* isolate), he suggested that it was by the decarboxylation of aconitate. This was supported in tracer studies of Bentley and Thiessen in *A. terreus* (1957a, b & c) who discovered the presence of a cis-aconitate decarboxylase (CAD) enzyme which was proposed to catalyse IA production. Three different pathways for the production were proposed by researchers over decades and they were investigated by Jaklitsch *et al.* in 1991

and by Bonnarne et al. in 1995. Both teams arrived at the conclusion that the pathway via aconitase and CAD was most likely. Later the CAD enzyme was isolated by Dwiarty et al. (2002), and Kanamasa and co-workers identified the gene and used it to transform *Saccharomyces cerevisiae* in 2008, thus proving that the gene coded for the CAD enzyme and that the CAD enzyme catalysed the production of itaconic acid (**Figure 4.9**).

It is now widely accepted that IA is produced as a result of the tricarboxylic acid cycle, through the decarboxylation of aconitic acid catalysed by CAD enzyme. It has also been proven that unlike the preceding enzymes in the biosynthetic process, such as aconitase and citrate synthase which are located in the mitochondria, CAD enzyme is located in the cytosol. However, a residual level of aconitase and citrate synthase activity is also found in the cytosolic fraction. The proposed mechanism is that *cis*-aconitate is transported via the malate–citrate antiporter into the cytosol (Jaklitsch *et al.*, 1991).



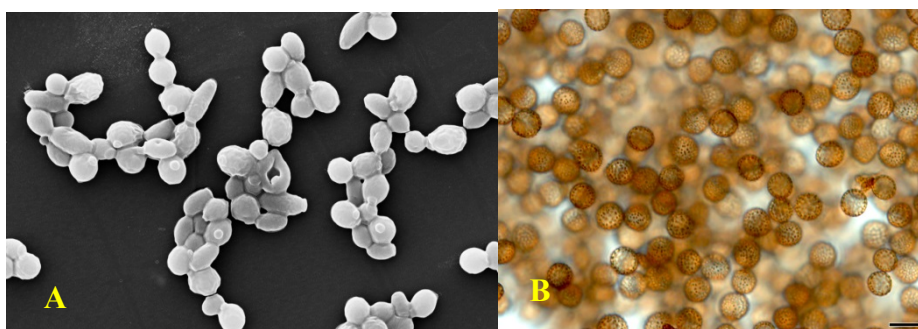
**Figure 4.9:** Simplified schematic of the biosynthesis of itaconic acid (adapted from Ahmed El-Imam and Du, 2014).

#### 4.1.2.5 Commercial Itaconic Acid Production

Prior to the 1960s, itaconic acid was mainly produced by the dry distillation of citric acid and treatment of the resulting anhydride with water (Lucia *et al.*, 2006). It was also produced by heating the calcium aconitate solution produced in the cane sugar refining process. The fermentative production of itaconic acid is mainly by submerged fermentation in large vessels by *A. terreus* in conditions similar to those of citric acid production by *A. niger* (Ruijter *et al.*, 2002). These conditions include the availability of an excess of readily metabolisable carbon source, high levels of dissolved oxygen, limiting amounts of metal ions and an ammonium-based nitrogen source. The exact details of the processes are proprietary but the general procedures are common knowledge (Ruijter *et al.*, 2002).

#### Microorganisms

In order to find the ideal industrial/commercial organism for IA production, several attempts have been made to find other microorganisms that are not as sensitive to particular fermentation conditions (e.g. substrate impurities) as *A. terreus*, or which produce a more favourable end-product composition. Some alternative natural producers have been investigated. For example, while screening strains of *Ustilago* sp for the production of ustilagic acid, IA was found in the fermentation broth of an *Ustilago zeae* strain which in later experiments was found to produce about 15 g IA/l (Haskins *et al.*, 1955). Among the filamentous fungi, other members of the ustilaginales have also been shown to produce IA. Different *Ustilago* species were tested by the Iwata Corporation (Japan) and a yield of 53 g IA/l was recorded within 5 days by *U. maydis* from glucose (Tabuchi and Nakahara, 1980; Tabuchi *et al.*, 1981). Since growing filamentous fungi may cause particular problems in bioreactors, yeasts were also tested for IA production. A strain of yeast identified as *Candida albicans* (**Figure 4.10**) was obtained after screening and subsequent mutation which produced about 35% yield of IA from glucose in 5 days (Tabuchi *et al.*, 1981), and efforts have continued to get increase yields from better IA producing strains. Non-natural producers such as *Escherichia coli* and *A. niger* have also been investigated, but yields were low (Li *et al.*, 2011).



**Figure 4.10:** Itaconic acid producing microorganisms. A: *Candida albicans*; B: Spores of *Ustilago maydis*. Picture credit: A: [www.collections.daff.qld.gov.au](http://www.collections.daff.qld.gov.au) ; B: [www.biotrans.uni.wroc.pl](http://www.biotrans.uni.wroc.pl)

Strain improvement has also been carried out. In Japan, mutation of *A. terreus* with N-methyl-N'-nitro-N-nitrosoguanidine resulted in one strain that produced 82 g IA/l after 7 days, a 1.3-fold increase compared to the parent (Yahiro *et al.*, 1995). Several genetically modified producers of itaconic acid have also been reported which have mainly involved the transfer of CAD into other microorganisms (Klement and Büchs, 2013), although these have yielded low yields. In addition it has been suggested that a supposed mitochondrial transporter discovered in the IA gene cluster (Li *et al.*, 2011) could be a specific transporter for *cis*-aconitate and this will likely be a focus for future genetic modification.

### Feedstock

The best yields of IA are achieved with glucose or sucrose as feedstock, but these are expensive and are not sustainable as the process will compete with the food uses of these sugars. Several other carbon sources have also been tested (**Table 4**), including the use of sorghum starch (although not the waste bran) (Petrucchioli *et al.*, 1999). However, most of these feedstock are very variable and IA production is sensitive to several medium components, including nitrogen and trace metals which means that, in order to get reproducible high productivities, the substrate quality has to be improved by any of various pretreatment methods before or during fermentation, in lieu of using refined starting materials.

The feedstock/substrate of choice depends on prevailing conditions of availability, plant location, market situation, energy costs, petroleum prices and other factors. The most frequently used substrates are beet or sugarcane molasses, starch, sugars such as sucrose and

glucose and glycerol. Interestingly, other organic acids have also been used as carbon sources, such as the relatively new approach of using citric acid as a precursor in a membrane reactor. With the price of citric acid being significantly less than that of itaconic acid, this method is likely to not only be easier, but also quite economical (Bressler and Braun, 2000), however, there is no indication that this process is being employed industrially.

### **Immobilisation**

Immobilization of microbial cells in biological processes can occur either as a natural phenomenon or through artificial process. It usually involves the entrapment of microbial cells within polymeric matrices. While the cells will exhibit significant growth in their natural habitat, the artificially immobilized cells are only allowed restricted growth. Immobilisation eliminates most of the constraints faced with the free-cell systems which include low cell density, nutritional limitations, and batch-mode operations with high down times (Ramakrishna and Prakasham, 1999).

Immobilisation can also be carried out to improve biomass handling and productivity. Various matrices have been used including a porous disk reactor system (Ju and Wang, 1986), silica-based material, alginate, polyurethane cubes (Kautola *et al.*, 1985; Kautola *et al.*, 1991), structural fibrous network of pawpaw trunk wood (Iqbal and Saeed, 2005) and polyacrylamide gel which yielded a particularly high productivity (Horitsu *et al.*, 1983).

### **Fermentation Process**

The fermentation process requires an excess of simple, readily metabolisable sugars such as glucose and sucrose as carbon source, which can be found in substrates such as molasses, glucose syrups, hydrolysates of starch etc. In addition, the process requires a low starting pH of 3-5, low phosphate to retard mycelial growth, operating pH of 2.2-3.8, moderate levels of nitrogen and trace metal (zinc, copper and iron), high initial glucose concentrations of 10-20%, a temperature of 37-40°C and extensive aeration (Lockwood and Reeves, 1945; Nubel and Ratajak, 1962; Willke and Vorlop, 2001). The optimum ranges of the various parameters of itaconic acid fermentation are presented in **Table 4.1** (Ahmed El-Imam and Du, 2014).

A patented process was developed which involved the fermentation molasses to IA by *A. terreus* producing up to 71 g/l from 150 g/l of sucrose (Nubel and Ratajak, 1962). Stock

cultures of the fermenting organism, usually *A. terreus* are germinated in large volumes of molasses medium (containing around 15% sucrose). The medium is held at 33°C and continuously aerated over a period of 18 hours to ensure germination into mycelia with the desired characteristics. Inoculation is then carried out using inoculum in the ratio of one to five of the fermentation medium, while the temperature is maintained at 45°C over 24 hours during which time the pH will drop from about 4.1 to 3.1. The fermentation is allowed to proceed for a further 2 days while the pH is maintained at 3.8 by the addition of an alkali such as sodium hydroxide or ammonia.

**Table 4.1:** Fermentation conditions employed and yields obtained (in terms of IA concentration in g/l in the final culture medium) as reported in the scientific literature (Ahmed El-Imam and Du, 2014).

MICROORGANISM	SUBSTRATE	TEMP. (C)	TIME (days)	pH	AERATION (RPM)	CONCENTRATION (g/l)	REFERENCE
<i>A. terreus</i>	Jatropha cake	R.T	9	1.5	400	48.7	(Ahmed El-Imam <i>et al.</i> , 2013)
<i>A. terreus</i> CECT 20365	Olive & beet waste	30	5	5.5	N/A	44	(Nikolay <i>et al.</i> , 2013)
<i>A. terreus</i> XL-6	Potato starch	36.7	5	2.4	197	N/A	(He <i>et al.</i> , 2012)
<i>A. terreus</i>	Glucose, glycerol	37	6	4.5	200	26.9 & 30.2	(Vassilev <i>et al.</i> , 2012)
<i>A. terreus</i> DSM23081, NRRI 1960 & 1963	Glucose	33	7	3.1	120	87-91	(Kuenz <i>et al.</i> , 2012)
<i>A. terreus</i> MJL05	Glycerol	30	8	2.4	N/A	27.6	(Juy <i>et al.</i> , 2010)
<i>A. terreus</i> TN484-M1	Sago starch	40	6	2.0	295	48.2	(Dwiarti <i>et al.</i> , 2007)
<i>A. terreus</i>	Jatropha cake	32	5	3.5	200	24.5	(Rao <i>et al.</i> , 2007)
<i>A. terreus</i> 282743	POME	30	5	5.8	150	5.76	(Jahim <i>et al.</i> , 2006)
<i>A. terreus</i>	Glucose		7	3.0	150	23.5	(Iqbal and Saeed, 2005)
<i>A. terreus</i> M-8	Starch hydrolysate	35	4	2.5-2.8	N/A	55	(Tsai <i>et al.</i> , 2001)
<i>A. terreus</i>	Citric acid	30	2	6.4	200	N/A	(Bressler and Braun, 2000)
<i>A. terreus</i> NRRL1960	Various starches	35	6	3.4	500	18.4	(Petruccioli <i>et al.</i> , 1999)
<i>A. terreus</i> NRRL1960	Sucrose	30	3	-	150	59	(Lockwood and Reeves, 1945)
<i>A. terreus</i> TN-484-M1	Glucose	37	6	2.0	220	82	(Yahiro <i>et al.</i> , 1995)
<i>A. terreus</i> TKK200-5-1	Glucose	37	14	3.1	200	51	(Kautola, 1991)
<i>A. terreus</i> TTK 200-5-3	Sucrose	37	14	3.0	200	13.3	(Kautola <i>et al.</i> , 1989)
<i>A. terreus</i>	Glucose and Sucrose	30	14	3.5	N/A	54	(Lai <i>et al.</i> , 2007)
<i>A. niger</i>	Glucose	33	10-13		180	1.4	(Blumhoff <i>et al.</i> , 2013)
<i>A. niger</i>	Glucose	33	5-6	2.3	N/A	2.5	(Li <i>et al.</i> , 2012)
<i>Ustilago maydis</i>	Glucose	34	5	3.0	180	29	(Rafi <i>et al.</i> , 2012)
<i>Ustilago maydis</i> MB215	Glucose	30	-	6.8	300	20	(Klement <i>et al.</i> , 2012)
<i>Pseudozyma antarctica</i>	Sugars	28	10	5.0	1000	30	(Levinson <i>et al.</i> , 2006)

## Downstream Processing and Product Recovery

A simple recovery method was described by Lockwood in 1975. This involves the removal of mycelia and other solids by filtration, evaporation under acidic conditions followed by cooling and crystallisation. This process provides an industrial grade of IA while if higher purity IA is required, the evaporate is treated with activated charcoal and anion exchangers (Willke and Vorlop, 2001). Itaconic acid can also be recovered by precipitation as insoluble salts which are then redissolved using alkali (Willke and Vorlop, 2001).

In a bid to reduce the costs, newer recovery methods have been developed which could potentially considerably improve the economics of commercial itaconic acid production. Notably, adsorption of IA onto ion-exchange resins in fixed-bed columns have been reported to be a more efficient recovery process (Magalhães Jr *et al.*, 2015) with the added advantage of integrating the fermentation and recovery processes. However, present day yields of IA amounting to just over 80 g/l have remained much lower than the theoretical maximum of 240 g/l, especially when compared with citric acid yields of over 200 g/l (Steiger *et al.*, 2013). **Table 4.1** shows some reported final yield values.

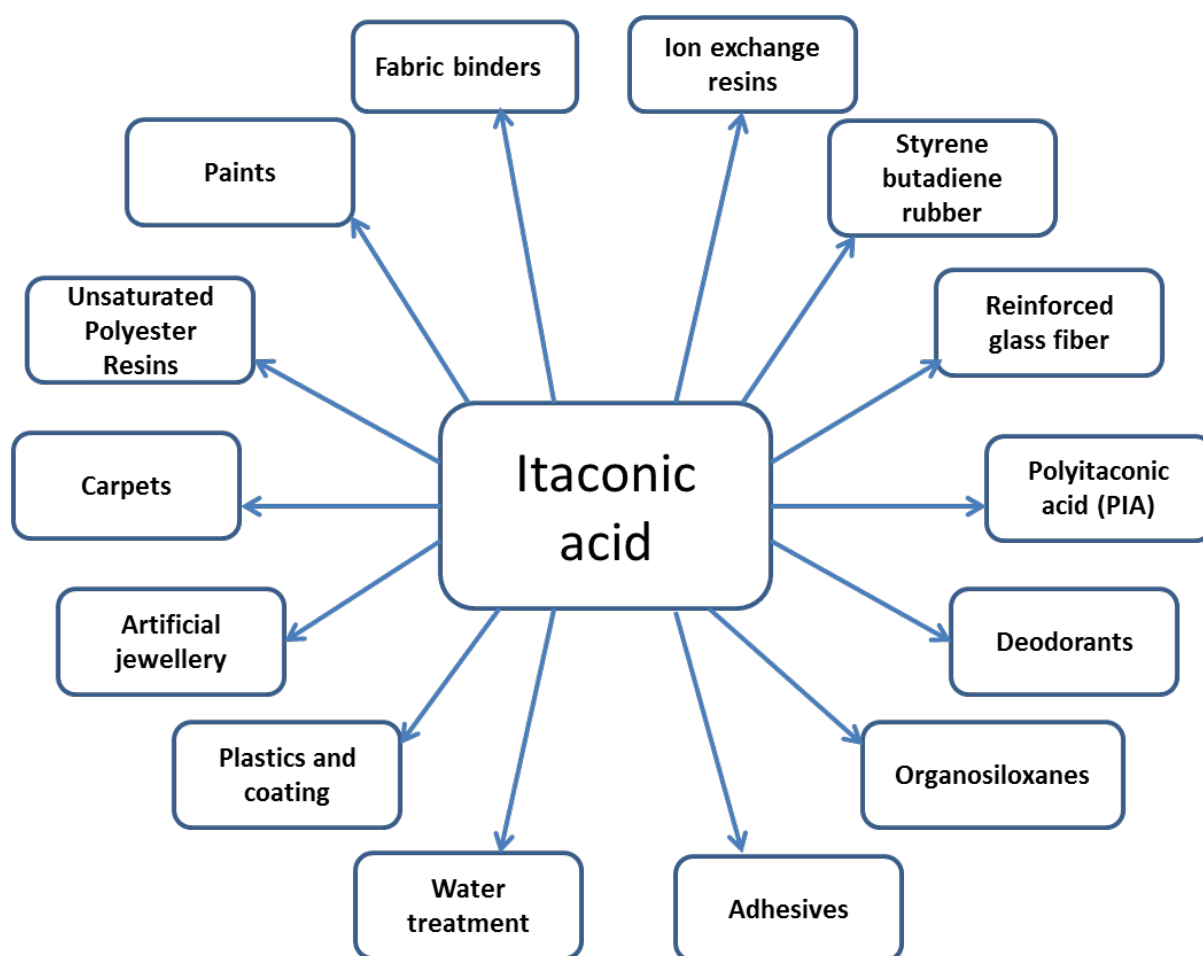
### 4.1.2.6 Industrial Applications of Itaconic Acid

There are two distinct routes through which itaconic acid is processed into polymers: radical self-polymerisation into polyitaconic acid, and step polymerisation to produce an unsaturated polyester and a sugar-derived polyol (Van Heiningen, 2006). Potential applications of IA are numerous and they are illustrated in **Figure 4.11**. However, the market is currently limited by the relatively high cost of both the chemical and the fungal fermentation-based processes. The current major uses are:

### Polymer Manufacture

The major application of itaconic acid is in the plastics industry. Itaconic acid is a substituted methacrylic acid and is thus a functionalized analogue of acrylic acid, the simplest conjugated alkenoic acid. Like acrylic acid, IA is able to take part in addition polymerization, giving polymers with many free carboxyl groups that confer advantageous properties on the polymer. These polymers are used in the paper industry for the production of various types of paper including wall paper, and also adhesives (Lucia *et al.*, 2006).





**Figure 4.11:** Industrial applications of itaconic acid (Ahmed El-Imam and Du, 2014).

## Textiles

Acrylic lattices are supplemented with itaconic acid and used as non-woven fabric binders. A copolymer of IA and acrylonitrile is also easier to dye than many other polymers (Lucia *et al.*, 2006).

## Paints

Itaconic acid is used in the manufacture of emulsion paints, where it improves the adhesion of the paint (Lucia *et al.*, 2006) When IA is added at a level of 5% in acrylic resins, it imparts to the resins the ability to hold printing inks (van Balken, 2005).

## Medical/Pharmaceutical

Itaconic acid is used as a hardening agent in organosiloxanes for use in contact lenses. In addition, mono and esters of partly substituted itaconic acid possess analgesic properties while some display plant-growth related activities (Lucia *et al.*, 2006).

Itaconic acid yields are on the order of 50-60% of the theoretical maximum and with the current price of \$2/kg (Okabe *et al.*, 2009) it is an expensive monomer. The refined sugars used add considerably to the high cost of IA and it is estimated that it contributes up to a quarter of the total production cost (Kobayashi and Nakamura cited in (Okabe *et al.*, 2009)) Van Heiningen (2006) reports that the market price of itaconic acid in 2006 was around \$2,400/MT, while the cost of producing it from hemicelluloses was about \$400/MT (assuming a 50% mass conversion). This indicates that the use of biomass carbon sources for renewable itaconic acid production is potentially very profitable. The use of cheap biomass can thus improve the economics of itaconic acid production, consequently making it more competitive and increasing its uptake industrially.

Bioethanol is the most widely used biofuel for transportation globally with the attendant benefits that its use decreases the use of fossil fuels and thus decreases the environmental damages associated with fossil fuel use. Itaconic acid is a versatile organic acid which is currently produced by the fermentation of refined sugars thus making it expensive for widespread industrial use. Even though it is more environmentally prudent to utilise renewable fuels and chemicals to replace fossilised petroleum-based ones for use in transportation and manufacturing, the production of renewable products has been faced with a few challenges. Currently, the major hindrances are the question of the ethics of using food sources such as corn, sugar cane and refined sugars to produce fuels and chemicals (**Section 1.1.5**) which resulted in the focus on alternative plant raw materials namely biomass (**Section 1.2.6**). However the difficulty involved in overcoming recalcitrance in the major raw material, lignocellulosic biomass, and other attendant problems such as the production of inhibitors has made it difficult to economically and sustainably produce bioproducts (Section 2.6.4). In this chapter, the feasibility of the use of enzyme and dilute acid hydrolysates of sorghum bran for the fermentative production of value added products is investigated. The

first product to be investigated is the primary metabolite and fuel bioethanol, and the second is the organic acid and secondary metabolite, itaconic acid.

#### **4.1.3 Aims**

The aim of this chapter was to evaluate whether ethanol and itaconic acid can be produced by fungal fermentations from the glucose-rich hydrolysates of sorghum bran.

The specific aims of this chapter are to:

- Perform a screening of yeast species with different properties on all sorghum bran hydrolysates in order to determine their metabolic profiles and suitability for downstream biotechnological applications
- Conduct mini-fermentations with the best identified yeast strains to investigate ethanol outputs
- Perform a screening of the fermentative ability of several *Aspergillus terreus* isolates to establish baseline itaconic acid fermentation yields on sorghum bran hydrolysates
- Determine the effect of various media glucose compositions on yield of itaconic acid, given that itaconic acid production has been reported to require high initial sugar concentrations
- Determine the overall suitability of all the hydrolysates for ethanol and itaconic acid fermentation, and the most ideal hydrolysate.

## **4.2 Materials and Methods**

This section contains only materials and methods used specifically in this chapter, general materials and methods are listed in Chapter 2.

All reagents used were analytical or HPLC grade. All solutions were prepared in sterile distilled water unless otherwise stated. All preparations for HPLC were conducted entirely with reverse osmosis water.

### **4.2.1 Ethanol Production**

The materials and methods specifically used only in the initial fermentation of sorghum bran hydrolysates for ethanol production are presented here. See **Section 3.3.4** for details of the media used in ethanol fermentations.

#### ***4.2.1.1 Media***

##### **- Sorghum Bran Hydrolysate Agar (SBHA) Preparation**

The four hydrolysates prepared in **Chapter 3** namely: the white bran dilute acid hydrolysate, the red bran dilute acid hydrolysate, the white bran enzyme hydrolysate and the red bran enzyme hydrolysate (**Section 3.4.3**) were used for the screening tests and the initial fermentations. For spot plate screening assay, sorghum bran hydrolysate agar (SBHA) was prepared as follows. The pH of each of the four hydrolysates was adjusted to 5.5 with 5 M sulphuric acid or sodium hydroxide, and 1.5 % w/v bacteriological agar was added. The mixture was sterilised at 121°C for 15 min and then poured into sterile Petri dishes which were stored at 4°C pending use.

##### **- YPD Agar**

YPD agar was prepared by adding 1.5 % bacteriological agar to YPD medium prior to sterilisation at 121°C for 15 min. The medium was left to cool to about 50°C then aseptically poured into Petri dishes.

#### ***4.2.1.2 Microorganism Preparation***

##### **- Inoculum For Spot-Plate Assay**

For the spot plate assay, yeast cell suspensions (**Section 2.2.3**) were serially diluted ten-fold in cuvettes, using sterile reverse osmosis water. The cuvettes were covered with Nescofilm (Fisher Scientific, UK) and inverted severally to mix. The absorbances were read at 600 nm with sterile reverse osmosis water as the blank. The numbers of cells in the suspensions were then counted with a Neubauer haemocytometer and their concentrations adjusted with sterile

reverse osmosis water to obtain an OD<sub>600</sub> of 1.0 on a spectrophotometer (Greetham *et al.*, 2014).

#### **- Inoculum For Phenotypic Microarray (PM) Analysis**

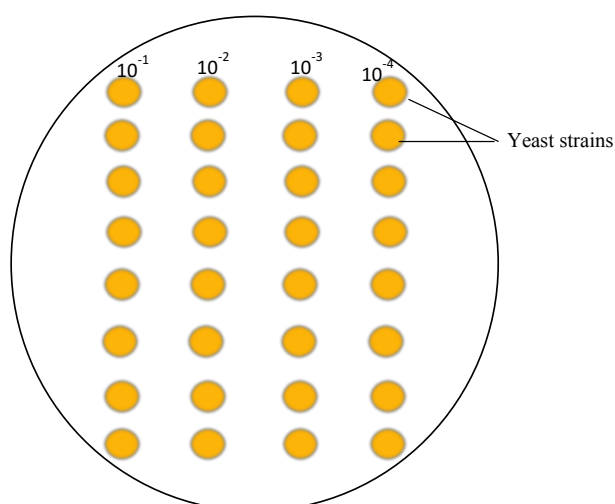
For the PM analysis, the transmittance for the cell suspension was measured using a Biolog turbidimeter (Biolog, CA, USA) and adjusted to 62% (approximately  $5 \times 10^6$  cells/ml) with sterile reverse osmosis water. Next 125 µL of the cells were mixed with 2.65 ml of IFY<sup>TM</sup> buffer (Biolog, CA, USA) and adjusted to a final volume of 3 ml with sterile reverse osmosis water (Greetham *et al.*, 2014).

#### **4.2.1.3 Screening of Yeast Strains**

These experiments were performed to determine the best-suited strain for sorghum bran hydrolysate fermentation.

#### **- Spot Plate Screening**

This was performed according to the method of Homann et al. (2000) modified by Greetham et al. (2014) with the further modification that in this work the medium used was not a YNB + glucose agar, but sorghum bran hydrolysate agar (SBHA) medium. The suspension of cells with OD<sub>600</sub> of 1.0 was serially diluted by ten fold stages and a 5 µL aliquot of each strain was aseptically transferred on to a plate of sorghum bran agar. Four plates of each of the four hydrolysates were prepared according to the arrangement illustrated in **Figure 4.12**. The plates were left undisturbed until the spots were completely dry, they were then transferred into carrier glass boxes and 2 plates each per hydrolysate incubated aerobically and anaerobically at 30°C for 72 hr.



**Figure 4.12:** Arrangement employed in inoculation in the spot-plate screening for tolerance of eight yeast strains to various sorghum bran hydrolysates on SBHA.

An anaerobic environment was achieved by including two AnaeroGen packs (Oxoid, U.K.) in an airtight incubation box, which turned white thus confirming an anaerobic environment was being maintained. After incubation, the plates were photographed in a Geldoc-It <sup>TM</sup> Imaging system under UV light using a VisionWorks LS Image acquisition and Analysis software (both manufactured by UVP LLC, USA).

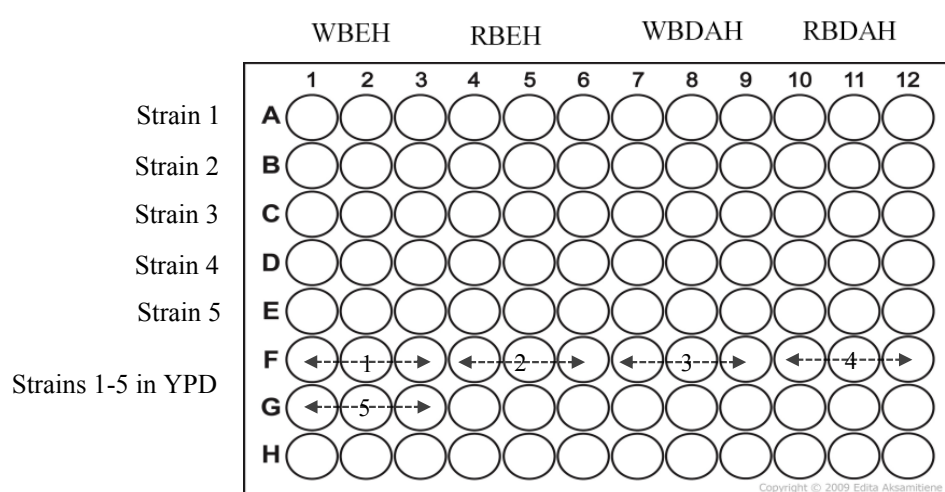
### - Phenotypic Microarray (PM) Analysis

This analysis provides an insight into the metabolic profiling of microorganisms within a 96-well plate system and successfully highlights differences in growth requirements, nutrient utilisation, sensitivity to toxins, and genetic diversity in most cell types (Greetham, 2014) In addition PM assay allows for the high throughput and rapid screening of microorganisms.

The PM assay was performed in an Omnilog instrument and utilised a redox sensitive tetrazolium dye which becomes irreversibly reduced upon detection of cellular metabolic output, and output from PM technology can be measured visually or quantified by reading the absorbance in each well (Greetham, 2014), with readings taken every 15 min.

These were carried out to evaluate the effect of the various hydrolysates on the cellular respiration of the yeast strains. Bespoke 96-well PM plates were utilised. Exactly 0.2  $\mu$ L of the tetrazolium dye was measured into each well. Next, 30  $\mu$ L of the white bran enzyme

hydrolysate was aliquoted into the first three wells. This was followed, also in triplicates, by the red bran enzyme hydrolysate, white bran dilute acid hydrolysate and red bran dilute acid hydrolysate respectively in the succeeding wells. This was repeated for four more rows in the same arrangement, thus preparing 5 rows for the test organisms. Next 90  $\mu$ L of the cell suspension (**Section 2.2.3**) was added to each well. Each row of 12 wells was thus inoculated with just one organism. A final 15 wells were filled with YPD medium as controls for the hydrolysates and inoculated with the yeasts in triplicates in the same order (**Figure 4.13**).



**Figure: 4.13:** Image of the bespoke 96-well plates showing the arrangement used to evaluate the effect of the hydrolysates on the cellular respiration of the yeast strains.

Anaerobic conditions were created using oxygen-absorbing packs (Mitsubishi AnaeroPak™ System) with an anaerobic indicator (Oxoid, Basingstoke, UK) and the plates were placed inside PM gas bags (Biolog, USA), sealed, transferred into the OmniLog reader and incubated for 72 hours at 30 °C. The OmniLog reader took photographs of the PM plates at 15 min intervals and converted the pixel density in each well to a redox signal intensity value reflecting dye reduction and thus, the metabolic activity of cells (Greetham *et al.*, 2014).

At the end of the run, the data were exported from the Biolog software onto Microsoft® Excel and the mean signal values were determined (Greetham *et al.*, 2014).

#### 4.2.1.4 Mini-Fermentation

From the findings of the results of the spot-plate and PM experiments, the best-performing strains were utilised in mini-fermentations of the various hydrolysates. Fermentations were performed in 25 ml volumes of hydrolysate at pH 5.0 in anaerobic conditions within Wheaton glass serum bottles (30 ml; Wheaton, USA) based on a method adapted from Quain et al. (1985) and Powell et al. (2003). The vessels were inoculated with the yeasts at a pitching rate of  $1 \times 10^7$  cells/ml and weighed to the nearest 0.1 mg. They were then placed on magnetic stirrer plates set at 120 rpm and placed in an incubator (MIR-253, Sanyo Electric Co., Japan) set at 30 °C. During fermentation, progress was monitored periodically by periodic weighing to observe weight loss resulting from carbon dioxide waste which was expelled through the Bunsen valves in the cap from the metabolism of carbohydrates in the growth medium. The fermentation was allowed to proceed until constant weight was observed over several time points and this was considered to be the conclusion of fermentation.

Ethanol yields were expressed as a function of the maximum that can be generated from the glucose consumed, based on the equation for the bioconversion of glucose to ethanol.



The theoretical maximum amount of ethanol that could be produced was calculated on a weight basis using the stoichiometric calculation:

$$\text{Yield (g/g)} = \frac{\text{Ethanol produced (g)}}{\text{Glucose consumed (g)}}$$

But from the equation for bioconversion of glucose above,

$$\text{Theoretical maximum ethanol possible (g/g)} = 2 \times \left\{ \frac{\text{Molecular weight of ethanol (g)}}{\text{Molecular weight of glucose (g)}} \right\} = 0.51 \text{ g/g}$$

So, if 0.51 g/g represents the theoretically possible maximum ethanol yield that can be produced, or 100 %, then



$$\text{Yield (as \% ) of Theoretical Maximum} = \frac{\text{Yield (g/g)}}{0.51} \times 100 \dots\dots\dots(5.1)$$

#### ***4.2.1.5 Time Course Fermentations***

Here fermentation was as described in **Section 4.2.1.4** samples were collected from the fermentation flasks at every 24 h and analysed for ethanol and residual glucose. Triplicate vials were terminated at each time point for ethanol determination and cell counts.

#### ***4.2.1.6 Cell Counts***

The number of cells in the fermentation broths were counted at specific time points over the duration of the fermentation. Equal volumes of the broths were mixed with 0.01% methylene blue solution and the number of live cells, which stained blue, determined microscopically.

### **4.2.2 Itaconic Acid Production**

This section contains the materials required and methods utilised in experiments to determine the suitability of the four sorghum bran hydrolysates for itaconic acid production. The media and materials are as described in **Section 2.1.4**.

#### ***4.2.2.1 Screening of *Aspergillus terreus* Isolates***

Initial screening fermentations were performed in duplicates with 46 *Aspergillus terreus* isolates for itaconic acid production on defined glucose medium as described in **Sections 2.1.4** and **2.2.4** over seven days. Later fermentation experiments were then conducted using the hydrolysates from sorghum bran (**Section 3.4.3**). Samples were then collected and analysed for itaconic acid by HPLC (**Section 2.2.6**) in technical triplicates to determine the best fermenting strains on both media.

#### ***4.2.2.2 Time Point Fermentations***

Three high-yielding strains from the screening test were employed in time point fermentations over nine days, using both the defined glucose medium and the sorghum bran hydrolysates. This experiment was performed in order to determine suitability of sorghum

bran hydrolysates for itaconic acid production, by comparing levels of production with the glucose medium control, and also to provide details of the time course trends of itaconic acid production during fermentation. The use of the defined glucose medium fermentation also served to verify that high itaconic acid yields could be obtained with the selected strains while providing a reference point to compare hydrolysate yields. The flasks were shaken to ensure continued aeration under conditions previously described (Section 2.2.4.2) as it has been reported that even a slight interruption of agitation can cause a cessation of itaconic acid production (Kuenz et al., 2012). Samples were frozen pending analysis for residual glucose and itaconic acid.

#### 4.2.2.3 Itaconic Acid Yields

Having established the optimum duration for the fermentations, six strains were then used to conduct fermentations, and the glucose consumed and itaconic acid produced at the end of the fermentation determined. This was conducted in order to verify the yields obtained from the screening test, and also establish the baseline yield of the theoretical maximum (%) for the strains on a reported defined medium (Kuenz *et al.*, 2012) and in the hydrolysates. It was anticipated that results would also provide data to allow selection of the best performing medium and strain having accounted for glucose utilisation.

The theoretical maximum based on glucose utilised was calculated similarly as in equation 5.1 thus:

$$\text{Theoretical maximum IA (g/g)} = \left\{ \frac{\text{Molecular weight of IA (g)}}{\text{Molecular weight of glucose (g)}} \right\} = 0.72$$

This makes the maximum IA that can be produced per gram of glucose consumed 0.72 g or 72 % (Clark and Blanch, 1997).

So, if 0.72 g/g represents the theoretically possible maximum itaconic acid that can be produced or 100 %, then

$$\text{Yield (g/g)} = \frac{\text{Itaconic acid produced (g/l)}}{\text{Glucose consumed (g/l)}}$$

$$\text{Yield (\% of Theoretical Maximum)} = \frac{\text{Yield (g/g)}}{0.72} \times 100 \dots \dots \dots (5.3)$$

#### 4.2.2.4 Enriched Hydrolysate Fermentation

An experiment was performed to compare yields of itaconic acid from sorghum bran hydrolysates and defined media with similar glucose contents. The glucose content of the DAH was thus raised artificially using a concentrated glucose solution to match the glucose concentration of the standard glucose defined media (**Section 2.1.4**). This experiment was performed in order to see if the hydrolysate contained components that might enhance or depress IA production relative to the standard glucose defined media when glucose levels were similar i.e. eliminating the variable of glucose concentration.

Time point experiments were first performed to determine IA yields and fermentation trends in these glucose-rich media. Afterwards, a batch fermentation was conducted to compare yields in all the media with similar glucose contents. The sorghum bran hydrolysates had a glucose content of 47 g/l, and were therefore enriched with sterile 400 g/l glucose solution up to a final concentration of 120 g/l to match that of the standard glucose defined media. The defined medium was also prepared in these concentrations, while leaving other components at their set concentrations as listed in **Section 3.1.4**. These media were then fermented with the selected *A. terreus* strains and samples analysed for itaconic acid production and fungal biomass generation.

#### 4.2.2.5 Fed-Batch Fermentations

This experiment was performed to investigate the effect of the addition of glucose at the point of exhaustion of fermentation broth glucose on itaconic acid production trend and yield. This experiment was conducted as it was hypothesised that a switch in metabolism might have occurred at this point, so supplementing with glucose at this point might have lead to direct conversion to itaconic acid. Concentrated glucose syrup (400 g/L) was aseptically added to fermentation flasks on the day determined from time point experiments to be the point of glucose depletion in hydrolysates, raising their glucose levels to a similar point as the

ongoing glucose control fermentation. Care was taken to minimise the length of the interruption to the process, and the fermentations were left to proceed for 11 days.

## **4.3 Results**

Compositional analyses had revealed that sorghum bran was a robust biomass material with potential for several bioconversion processes. The findings of various experiments performed to investigate the potential of hydrolysates produced from both varieties of sorghum bran to produce value-added products are presented below. For initial experiments growth trends were assayed mainly by visual means. In later experiments more detailed replicated work was performed to allow statistical analyses to validate results.

### **4.3.1 Ethanol Production**

Several ethanologenic yeast strains were investigated for their metabolic responses to sorghum bran hydrolysates and subsequently, their ethanol yields from fermentation.

#### **4.3.1.1 Screening Tests**

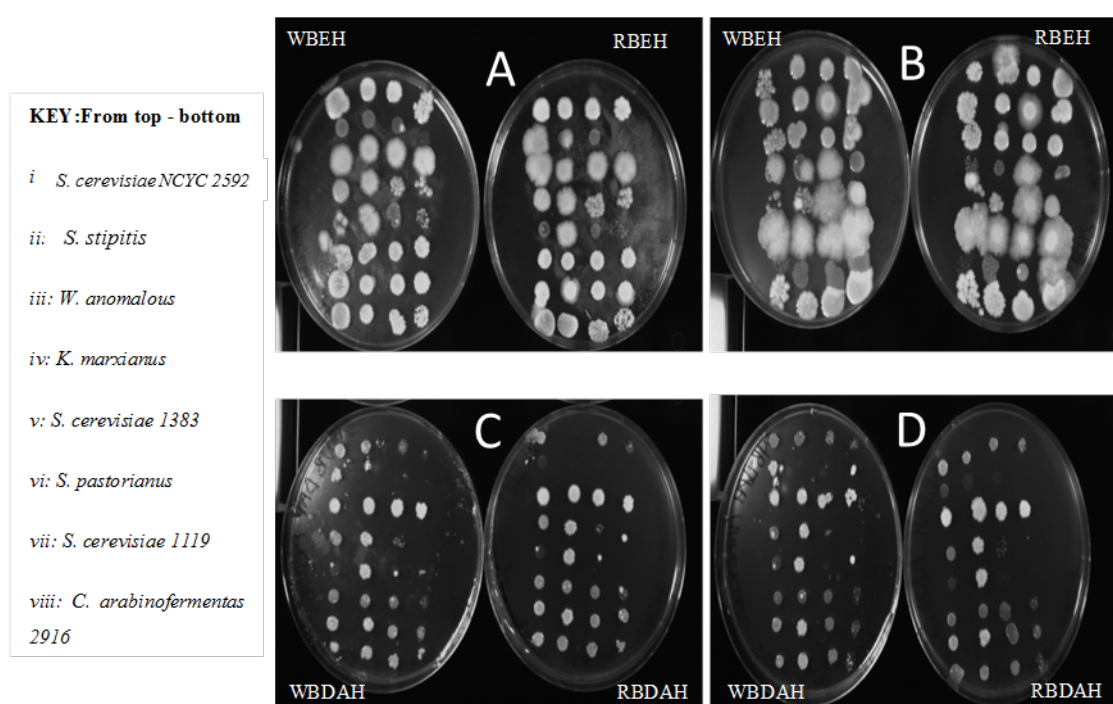
Screening tests were conducted to determine which yeast species were able to successfully metabolise and grow on the different hydrolysates in order to determine the best suited strains for subsequent fermentation work.

#### **- Spot-Plate Test**

The spot plate tests compared the ability of several ethanologenic yeast species to grow on agar media prepared from hydrolysates obtained from the two bran types (RB and WB) and also produced by two different methods (dilute acid and enzyme hydrolyses). They therefore revealed which organisms would be able to tolerate and grow on the hydrolysates i.e. their robustness with respect to tolerance of the complex nature of the substrates.

It was observed that all the yeast strains were able to tolerate the different types of media and show growth. However, *S. stipitis* and *S. cerevisiae* 1383 appeared to grow more slowly than the rest (**Figure 4.14**). The colony sizes decreased in some strains as serial dilutions were

plated, while in others including *S. cerevisiae* NCYC 2592 and *W. anomalous* the colony size was maintained despite the dilutions i.e. the  $10^{-4}$  spot appeared to be of the same size as the original OD = 1 spot. Regarding the effect of hydrolysate type, all strains grew more vigorously on agar media made from the enzyme hydrolysates than on the acid hydrolysates, which was correlated with the lower glucose content of the latter (approximately 54 g/l compared to 33.6 g/l, respectively).



**Figure 4.14:** Spot-plate screening tests showing typical pattern of eight species of yeast growing on agar plates prepared from four different types of sorghum bran hydrolysates and viewed under UV light with a GelDoc Imaging system (note NCYC 2592 is a *S. cerevisiae* strain). A and C: Aerobic incubation; B and D: Anaerobic incubation. Spots comprise yeast cells in dilutions of OD<sub>600</sub> = 1 stock from L-R:  $10^{-1}$  –  $10^{-4}$ . WBEH = white bran enzyme hydrolysate; RBEH = red bran enzyme hydrolysate; WBDHAH = white bran dilute acid hydrolysate; RBDHAH = red bran dilute acid hydrolysate.

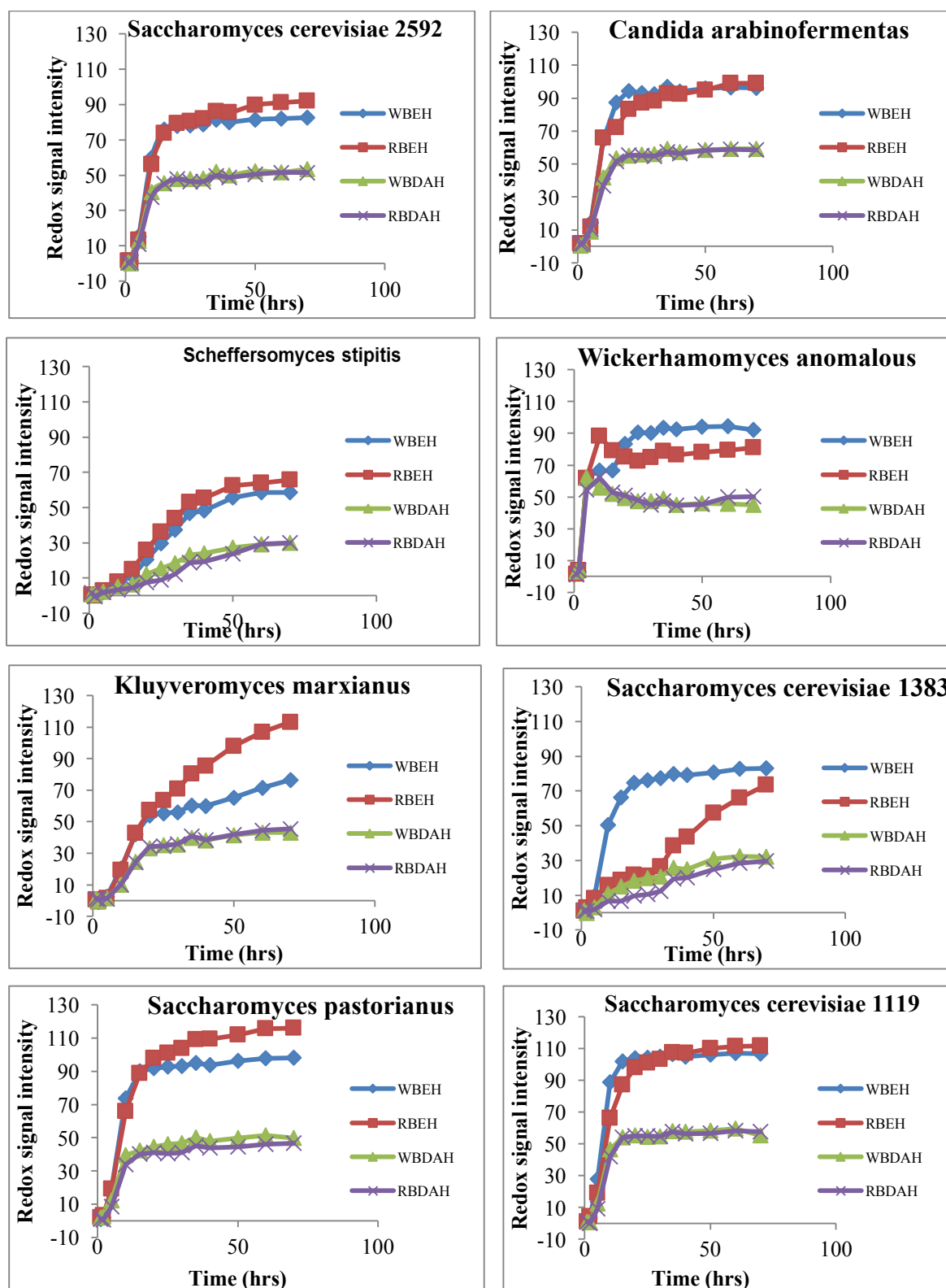
Under aerobic conditions, the organisms grew in more compact circles while a more swarming phenotype was observed in plates incubated anaerobically, particularly in the richer enzymatic hydrolysates (**Figure 4.14**). However, it was observed that there was no difference between the growth of the yeasts on the white or red bran hydrolysates, regardless

of how it was produced, or whether grown under aerobic or anaerobic conditions indicating that the brans did not differ in their ability to support the growth of the yeasts.

#### **- Phenotypic Microarray Analysis**

The results of the spot-plate analysis gave a preliminary indication of possible differences between yeast strains but could not be solely relied on as a screening test. Consequently a phenotypic microarray (PM) analysis was also performed. PM analysis was carried out by growing the strains on the four hydrolysates and then the cells' metabolic activities were assessed by redox signal intensity (**Figure 4.15**).

The metabolic activities of the yeasts were found to vary considerably between strains and between hydrolysates, even though the growth pattern was similar, with all strains exhibiting a short log phase of growth and eventually plateauing off into a lag phase. Similar to the observation with the spot plates, the enzyme derived hydrolysates supported a higher metabolic output than the dilute acid ones. *S. pastorianus*, *K. marxianus* and *S. cerevisiae* 1119 appeared to be the most metabolically active as evidenced by the intensity of their redox signals while the lowest metabolic activity was observed in *S. stipitis*. White and red bran dilute acid hydrolysates showed a similar pattern in supporting growth of all the yeast strains. By contrast, some variation was observed on the enzyme hydrolysates on which *S. stipitis* and *K. marxianus* grew better on RBEH than on WBEH, while the reverse was true with *W. anomalous* and *S. cerevisiae* 1383.

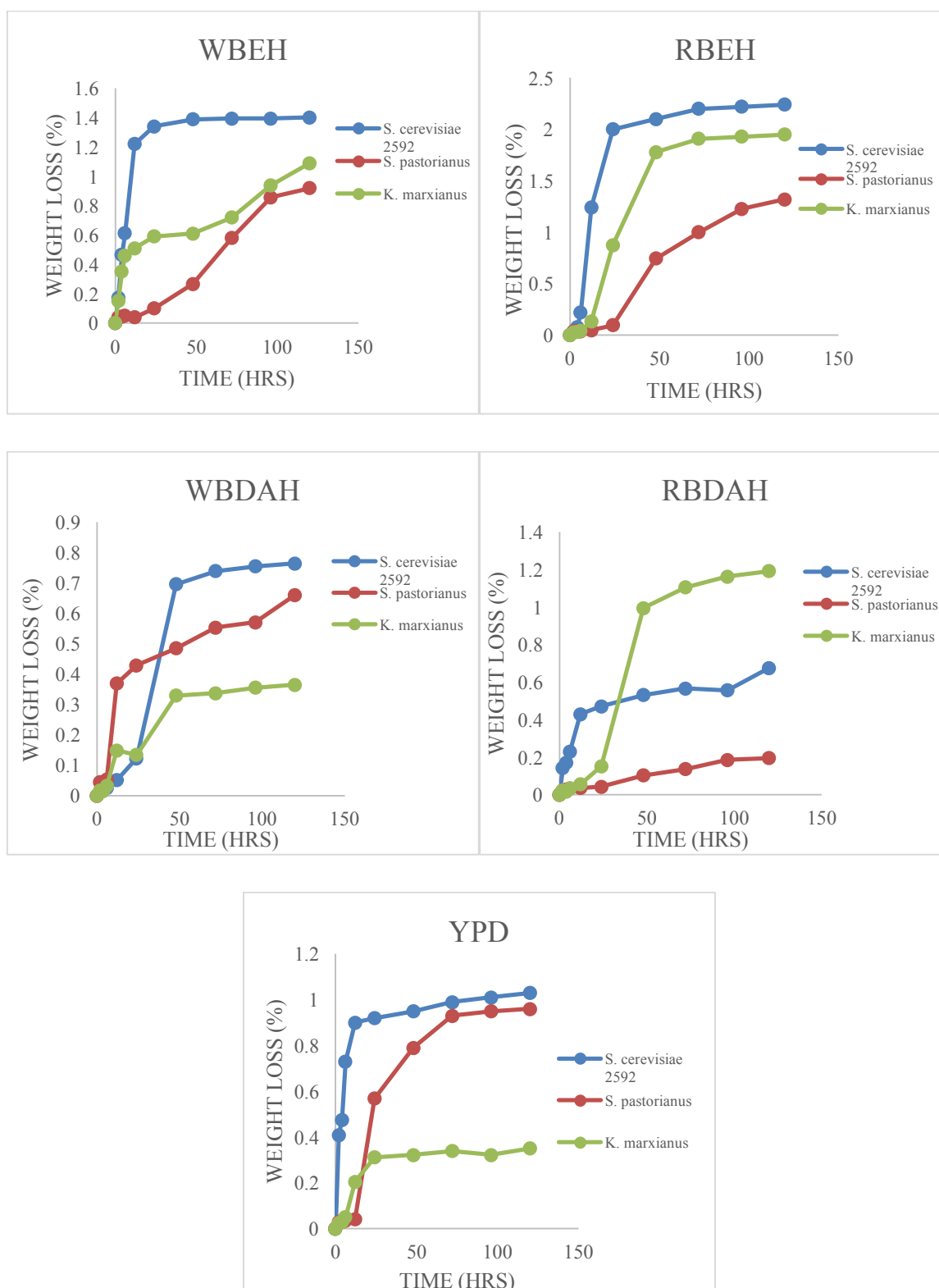


**Figure 4.15:** Phenotypic microarray analysis showing metabolic activity of 8 yeast strains, as judged by redox signal intensity, cultivated on four sorghum bran hydrolysates and incubated anaerobically in an Omnilog system over 72 hr. Hydrolysate labelling as in **Figure 4.14**. values are means of triplicate observations.

#### **4.3.1.2 Mini-Fermentations**

The strains with the best metabolic profile on the hydrolysates in the PM analysis and that displayed good growth in the spot-plate tests, namely *K. marxianus*, *S. cerevisiae* NCYC 2592 and *S. pastorianus*, were then employed in mini-fermentations on red bran hydrolysates to evaluate their hydrolysate fermenting abilities, using YPD as a control medium. All of the media exhibited weight loss indicative of active fermentation (**Figure 4.16**). The enzyme hydrolysates showed consistently higher weight loss than those from the dilute acid pretreatments. The loss was most pronounced in the RBEH at over 2 % loss whereas the least weight loss observed was in WBEH with just under 0.8 % reduction in vessel weight. *S. cerevisiae* displayed the highest loss in mass in WBEH, RBEH and WBDAH, while *K. marxianus* exhibited most loss of weight in RBDAH, and *S. pastorianus* in YPD (**Figure 4.16**).





**Figure 4.16:** Weight loss trends for mini-fermentations of four sorghum bran hydrolysates and a glucose medium control by strains of three different yeast species. Hydrolysate labelling as in **Figure 4.14**. YPD = Yeast Peptone Dextrose medium. Values are average of triplicate measurements. Error bars omitted for clarity, but ranged from 0.05 – 0.21.

#### 4.3.1.3 Ethanol Yields

The weight loss observations indicate that the mini-fermentations had proceeded as desired and indicated which strain were most metabolically active, but to determine actual yields of ethanol and other metabolites, HPLC analysis was required.

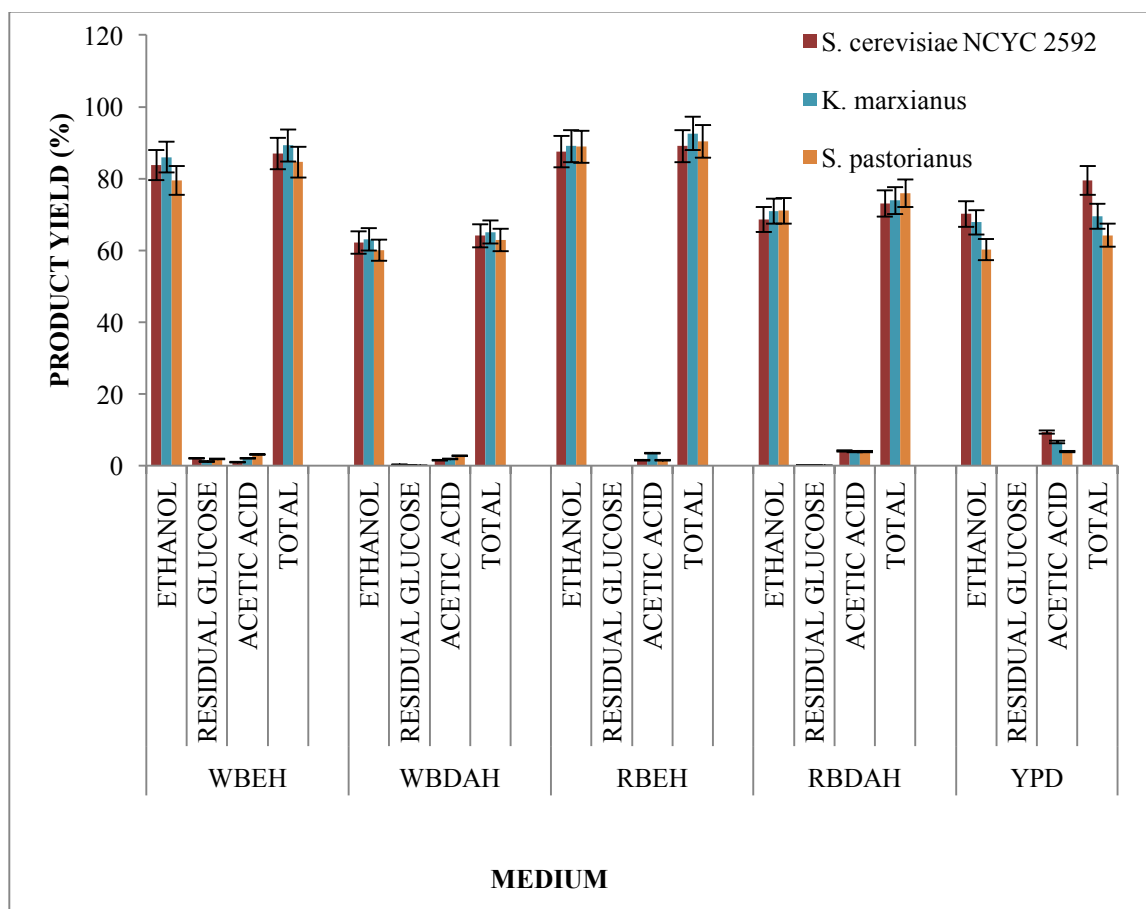
Fermentations were terminated when weights appeared to have stabilised and samples collected for analysis by HPLC. The ethanol concentrations obtained are presented in **Table 4.2** and *K. marxianus* was found to produce the highest amount of ethanol in all media. The highest ethanol concentrations by all strains was on RBEH while YPD gave the lowest. There was no significant difference between the ethanol yields from both enzyme hydrolysates ( $F = 817.33$ ;  $df = 4$ ;  $P = 0.0000$ ) nor was there a difference between yeast strains.

**Table 4.2:** Ethanol concentrations obtained from yeast fermentation of sorghum bran hydrolysates.

ETHANOL CONC (g/L)					
STRAIN	WBEH	WBDAH	RBEH	RBDAH	YPD
<i>S. cerevisiae</i> 2592	23.08 <sup>a</sup>	15.17 <sup>b</sup>	23.75 <sup>a</sup>	17.70 <sup>c</sup>	7.18 <sup>d</sup>
<i>K. marxianus</i>	23.66 <sup>e</sup>	15.38 <sup>f</sup>	24.35 <sup>e</sup>	18.29 <sup>g</sup>	6.94 <sup>h</sup>
<i>S. pastorianus</i>	21.89 <sup>i</sup>	14.65 <sup>j</sup>	22.53 <sup>i</sup>	18.32 <sup>k</sup>	6.16 <sup>l</sup>

<sup>a,b</sup> Values within rows bearing different alphabet superscripts are significantly different

The yields as a function of glucose converted to ethanol, and the main quantifiable by-product acetic acid, are shown in **Figure 4.17** below.



**Figure 4.17:** Ethanol yields from the fermentation of various types of sorghum bran hydrolysates and YPD (as a control medium) over 120 h. Values are the yields as a function (%) of the theoretical maximum possible (Section 4.2.1.4); the total (%) yields are a sum of yields of analytes presented. All values are average of triplicate measurements with the error bars indicating standard deviations.

Results indicate that fermentations were indeed completed as there was minimal glucose left in the broths. Comparing yeast strains, a one-way ANOVA test found no significant differences ( $F = 0.39$ ;  $df = 2$ ;  $P = 0.6825$ ) between the performances of the three strains tested on any of the media. *S. pastorianus* had the widest range of ethanol yields, producing the least 60.1 % in WBDAH and highest of 88.9 % in RBEH. *S. cerevisiae* NCYC 2592 was the lowest performing yeast in both red bran hydrolysates but performed best in the YPD producing over 70 % of the theoretical maximum ethanol. *K. marxianus* performed better than other yeasts in the RBEH, WBEH and WBDAH while *S. pastorianus* only performed marginally better in the RBDAH (by 0.14%) than *K. marxianus*, and *S. cerevisiae* NCYC

2592 only outperformed other strains in YPD. Thus, *K. marxianus* appeared to be the best suited organism for sorghum bran hydrolysate fermentation.

Comparing the different media, significant differences in ethanol yield were found when a multifactor ANOVA was performed ( $F = 142.44$ ;  $df = 2$ ;  $P = 0.0000$ ). Multiple range tests for ethanol yield were then performed to determine which pair of means were significantly different. Among the sorghum bran hydrolysates, the enzyme hydrolysates (WBEH and RBH) showed statistically higher ethanol yield than the dilute acid pretreatments (WBDAH and RBDAH) according to a multiple range test (**Table 4.3**). For example, *K. marxianus* produced 89 % of the theoretical maximum of ethanol on RBEH compared with 70.9 % with the RBDAH, with the WBEH also yielding more than the WBDAH. The RBEH provided better yields than the WBEH, but differences were found to be insignificant at  $P = 0.05$ .

**Table 4.3:** Multiple range test for ethanol yield (%) comparing the performance of the various media (data pooled for all different yeast strains).

<i>Contrast</i>	<i>Sig.</i>	<i>Difference</i>	<i>+/- limits</i>
RBDAH - RBEH	*	-18.26	2.62
RBDAH - WBDAH	*	6.19	2.62
RBDAH - WBEH	*	-12.88	2.62
RBDAH - YPD	*	4.15	2.62
RBEH - WBDAH	*	24.45	2.62
RBEH - WBEH	*	5.38	2.62
RBEH - YPD	*	22.41	2.62
WBDAH - WBEH	*	-19.06	2.62
WBDAH - YPD		-2.04	2.62
WBEH - YPD	*	17.02	2.62

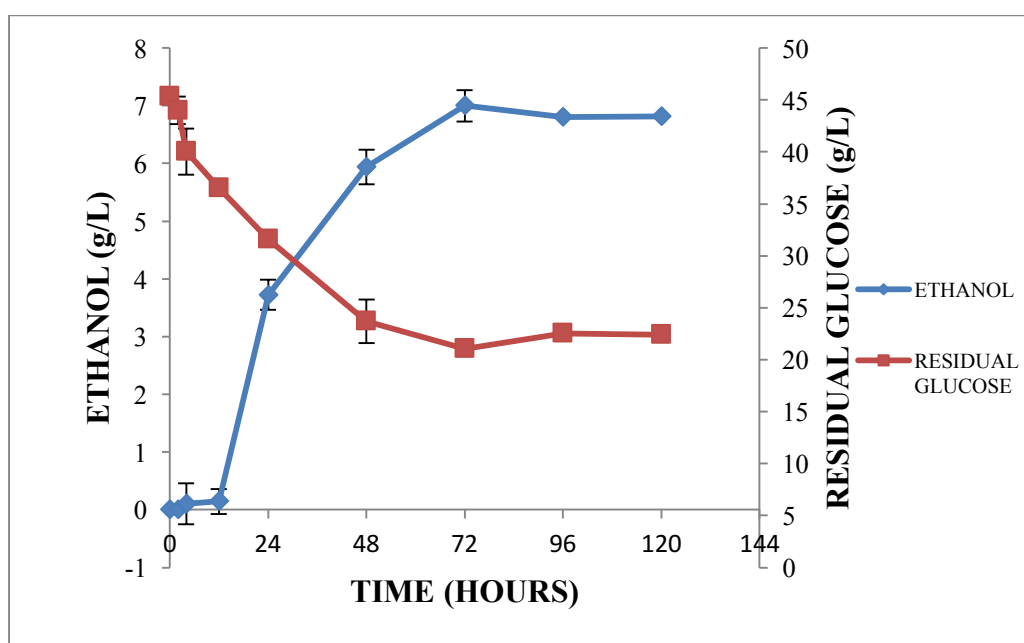
\* denotes a statistically significant difference.

It was surprising to observe that YPD had a relatively low ethanol yield with 70.1 % of the theoretical maximum ethanol produced by *S. cerevisiae* NCYC 2592 and even less by the

other strains. This was only better than the yield with WBDAH, where a maximum of 65.1 % was observed with *K. marxianus*. It was observed that there was no statistical difference between the WBDAH and YPD media in ethanol production (**Table 4.3**). The main by-product observed was acetic acid, with the highest (6.6 %) and lowest (1.5 %) amounts of the theoretical maximum being produced by *K. marxianus* in YPD medium and RBEH, respectively. The combination of acetic acid and ethanol production accounted for 96 % of the theoretical maximum that can be produced from the glucose available.

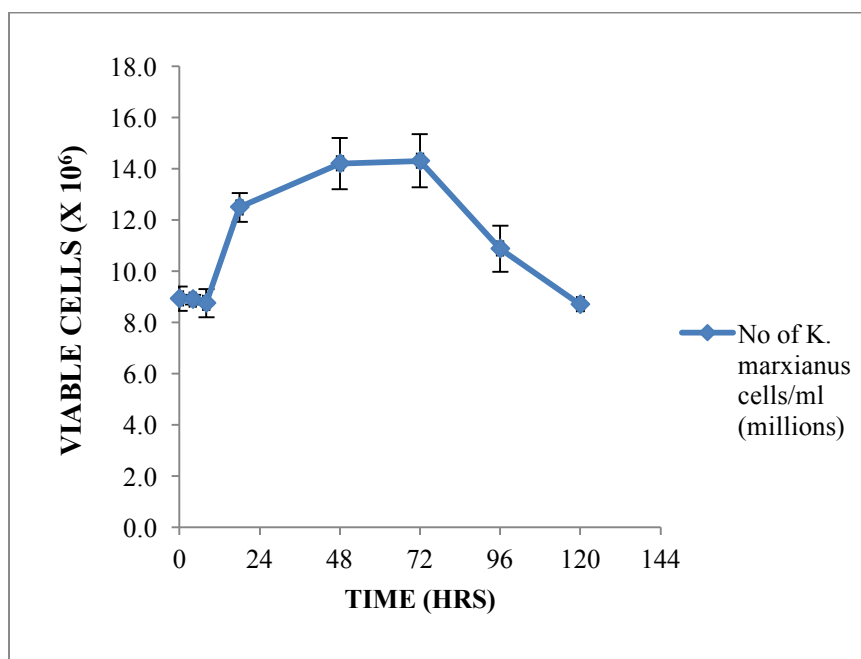
#### 4.3.1.4 Time Course Fermentation with Best Strain

The best-performing strain identified in the hydrolysate fermentations was *K. marxianus* and it was then solely employed in RBDAH fermentation to determine the time course of the fermentation, as well as the viability of the cells over time. It was observed that ethanol yields increased after a lag period of about 12 h during which 21.6 g of glucose was consumed with ethanol production peaking at 7 g/l before plateauing (**Figure 4.18**). This corresponds to 63.5 % of the theoretical maximum possible.



**Figure 4.18:** Time course fermentation with *K. marxianus* on red bran dilute acid hydrolysate over 120 hr. Values are means of triplicate fermentations. Error bars represent the standard deviation of the means.

The viable *K. marxianus* cells were counted after staining with methylene blue and the mean counts are shown in **Figure 4.19** below. It was observed that after 72 hr, the culture began to enter the decline phase as cells began to die off. This proceeded at a near exponential rate until the fermentation was terminated 48 h later.

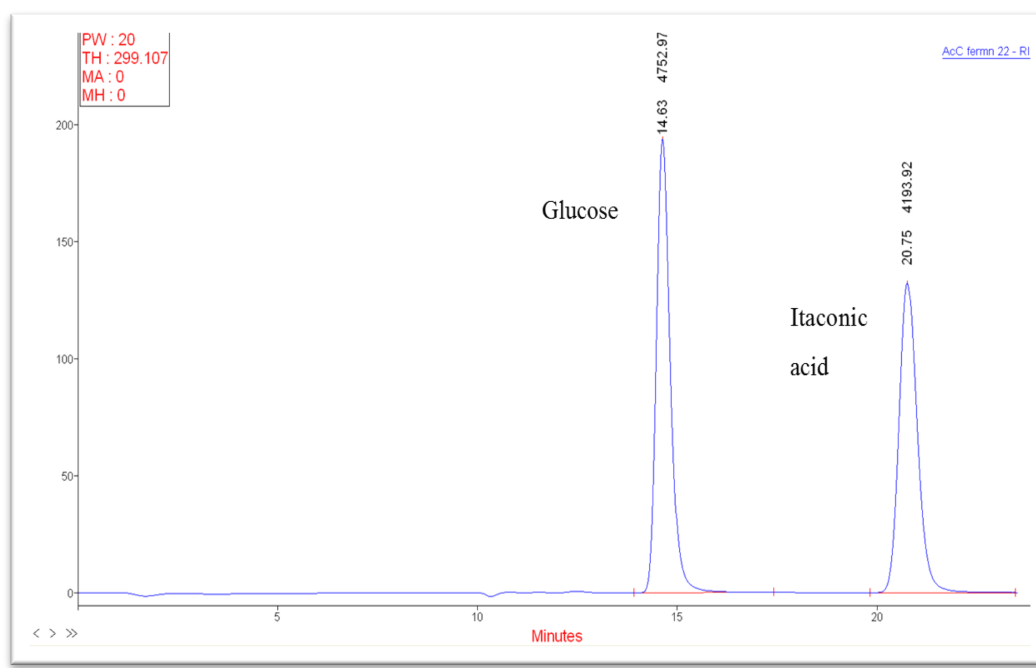


**Figure 4.19:** Counts of *K. marxianus* cells over the fermentation period (120 hr) observed microscopically after staining with methylene blue. Values are averages of triplicate counts (error bars are standard deviations).

#### 4.3.2 Itaconic Acid Production

By way of overview, 46 *A. terreus* isolates from the BDUN collection were firstly investigated for their IA producing capacities in a screening experiment on a defined liquid glucose medium adapted from that reported by Kuenz et al. (2012) for the submerged fermentative production of IA. The best strains were then selected and an experiment conducted in triplicates to accurately compare these. Next, time course experiments were performed to determine the trend of itaconic acid production by the strains of *A. terreus* over time in both glucose and sorghum hydrolysates. This information then informed the duration of subsequent fermentations. Subsequently, various parameters such as the effect of glucose content in the medium and glucose addition on the fermentation were investigated, as will

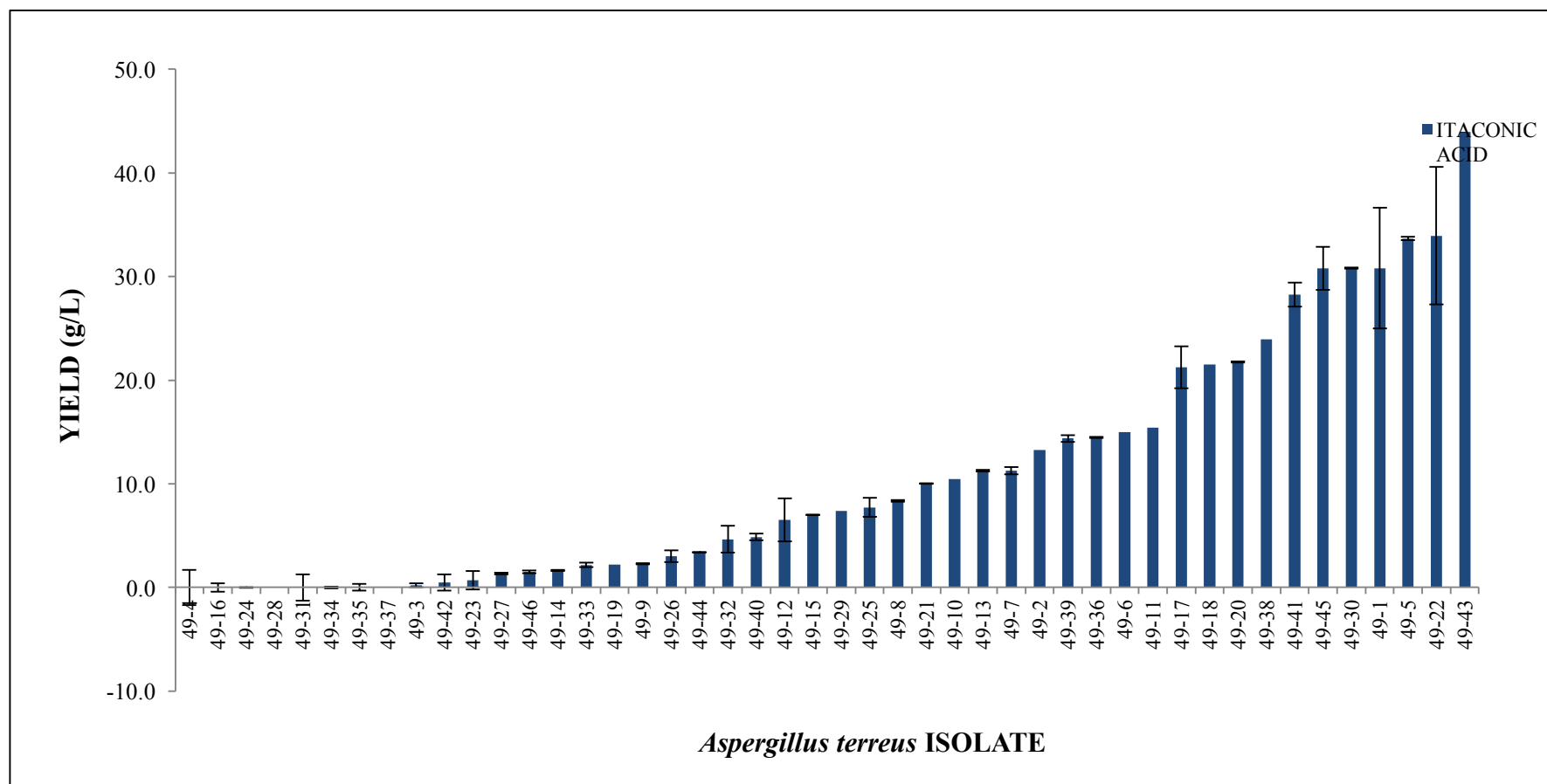
now be described. All HPLC analyses were conducted using Jasco HPLC equipment, with a typical itaconic acid fermentation chromatogram shown below (**Figure 4.20**).



**Figure 4.20:** Typical chromatogram illustrating resolving of reference solutions of glucose and itaconic acid when performing HPLC using a Hi-Plex H<sup>+</sup> column at 45°C with a mobile phase of 0.005N H<sub>2</sub>SO<sub>4</sub> at flow rate of 0.4 ml/min.

#### 4.3.2.1 Screening of *Aspergillus terreus* Isolates

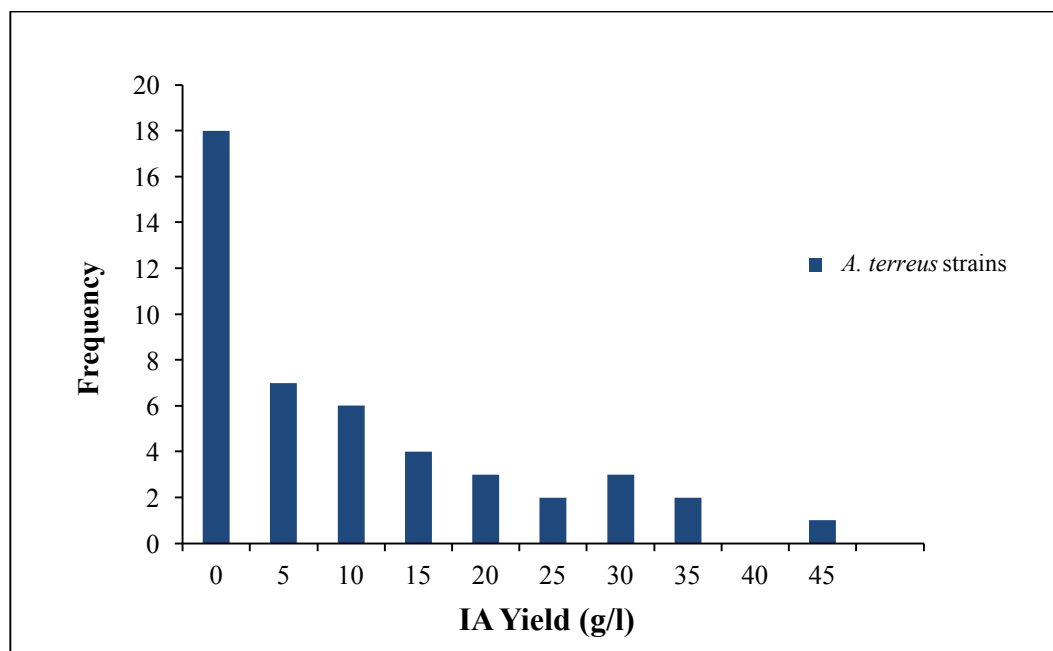
Several *A. terreus* isolates were screened for their itaconic acid production capacities by employing them in submerged fermentation of a defined glucose medium for seven days. This was aimed at identifying the strains with the best potential for economically competitive product formation. The findings of the screening are presented below (**Figure 4.21**).



**Figure 4.21:** Screening results of 46 *Aspergillus terreus* isolates investigated for itaconic acid production on defined glucose medium. Fermentations were performed at 37 °C and agitation rate of 200 rpm for 7 days. Fermentations were performed in biological duplicates and IA estimated in technical triplicates. Error bars are standard deviations.



To observe the trend of IA production in wild-type strains of *A. terreus*, a histogram was plotted and it was observed that production among the strains varied widely, ranging from 0.2 g/l – 43.9 g/l in 5.0 g/l intervals, in a distribution trend that was found to be skewed towards lower values (**Figure 4.22**). The majority of the strains were low producers with only strains 49-1, 49-5, 49-22, 49-30, 49-43 and 49-45 producing over 30.0 g/l of itaconic acid after 7 days. The yield from 18 strains ranged from 0.0 - 2.5 g/l which was the largest bin, followed by the 2.5 – 7.5 g/l bin with seven strains while the frequencies decreased with increasing bin sizes. There were no strains with IA yields in the 32.5 - 37.5 g/L bin and only strain 49-43 in the highest bin with 43.93 g/l yield.



**Figure 4.22:** Distribution of itaconic acid production by *Aspergillus terreus* isolates (frequency represents number of strains) in defined medium. The yield values shown (g/l) represent itaconic acid yields plotted as bin centres of the histogram.

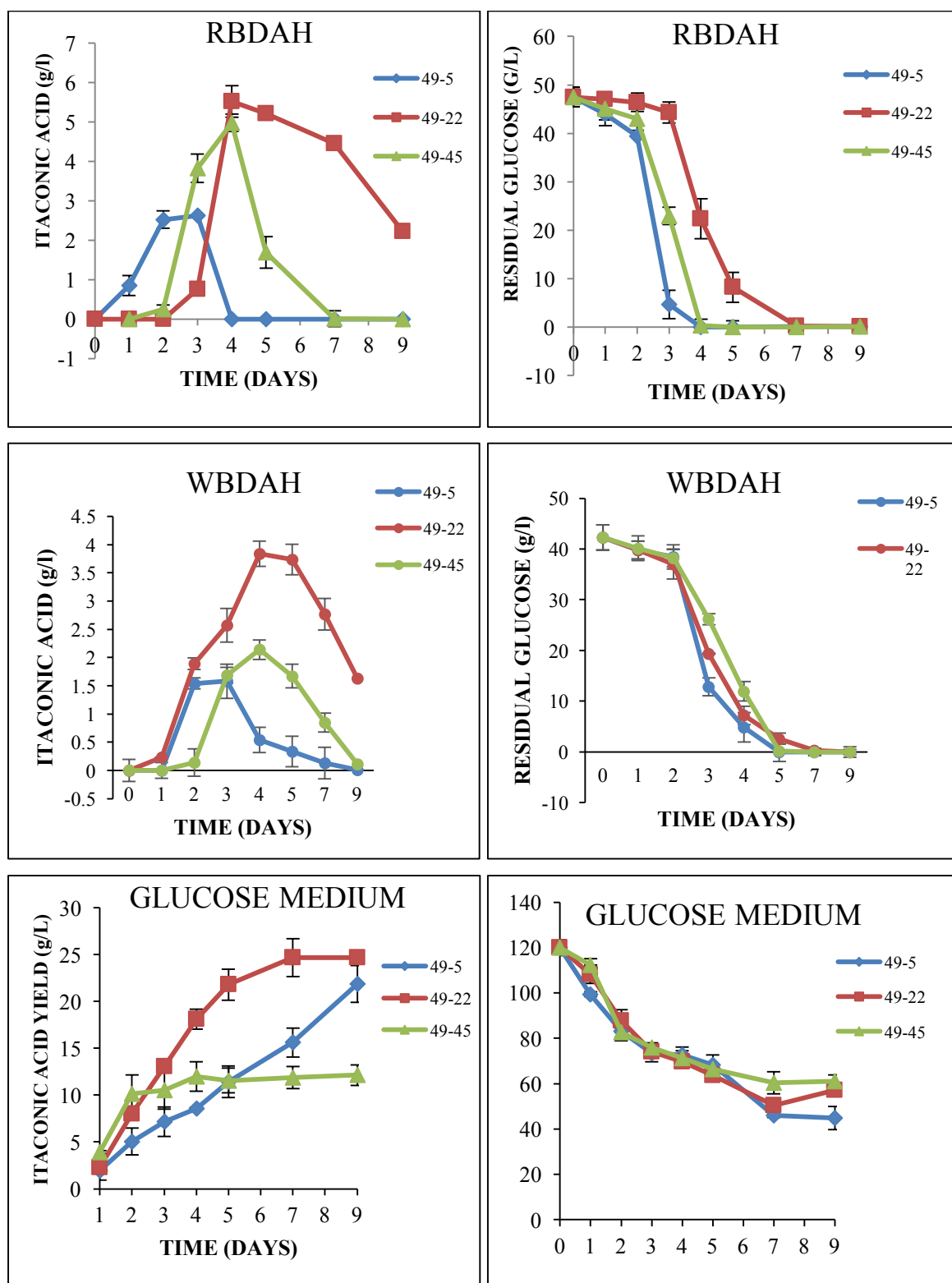
#### 4.3.2.2 Time Point Fermentation

Three representative *A. terreus* isolates (49-5, 49-22 and 49-45) were chosen for further analysis. Experiments were performed involving the fermentation of the WB and RB sorghum bran hydrolysates in order to determine peak production time, and the optimum length for itaconic acid production for subsequent fermentations (**Figure 4.23**). Control

fermentations were also performed in defined itaconic acid fermentation media adapted from Kuenz *et al.* (2012) alongside all bran hydrolysate fermentations. The glucose consumption trends were also observed by estimating the residual glucose at each time point. (**Figure 4.23**).

A key finding was that fermentation of both the white and red bran acid hydrolysates led to the production of itaconic acid by these isolates of *A. terreus*, demonstrating that sorghum bran provides a suitable feedstock for itaconic acid production. The fermentations commenced for all isolates after a brief lag phase of under 24 hr. Itaconic acid production peaked at day 4 in the RBDAH at 5.52 g/L, 4.97 g/L and 2.62 g/L by isolates 49-22, 49-45 and 49-5, respectively. Interestingly, it was observed that the itaconic acid produced did not then remain in the medium, but levels decreased (i.e. it was removed) after production peaked until there was no detectable itaconic acid in the system, except with 49-22 with 2.2 g/l by the end of the experiment. A similar trend was observed with the WBDAH where IA removal followed peak production at Day 4 with 3.84 g/l and 2.14 g/l for 49-22 and 49-45, except with 49-5 which peaked at Day 3 with 1.58 g/l. Glucose utilisation in both media was similar with all strains, and was completely exhausted by Day 7. In both media, strain 49-22 was the best itaconic acid producer (with 5.52 g/l which corresponds to 32.4 % of maximum possible); while RBDAH was the superior medium.

By contrast, no itaconic acid was detected when the same strains were grown on WBEH and RBEH media, nor was there visible mycelial growth after 9 days of incubation.

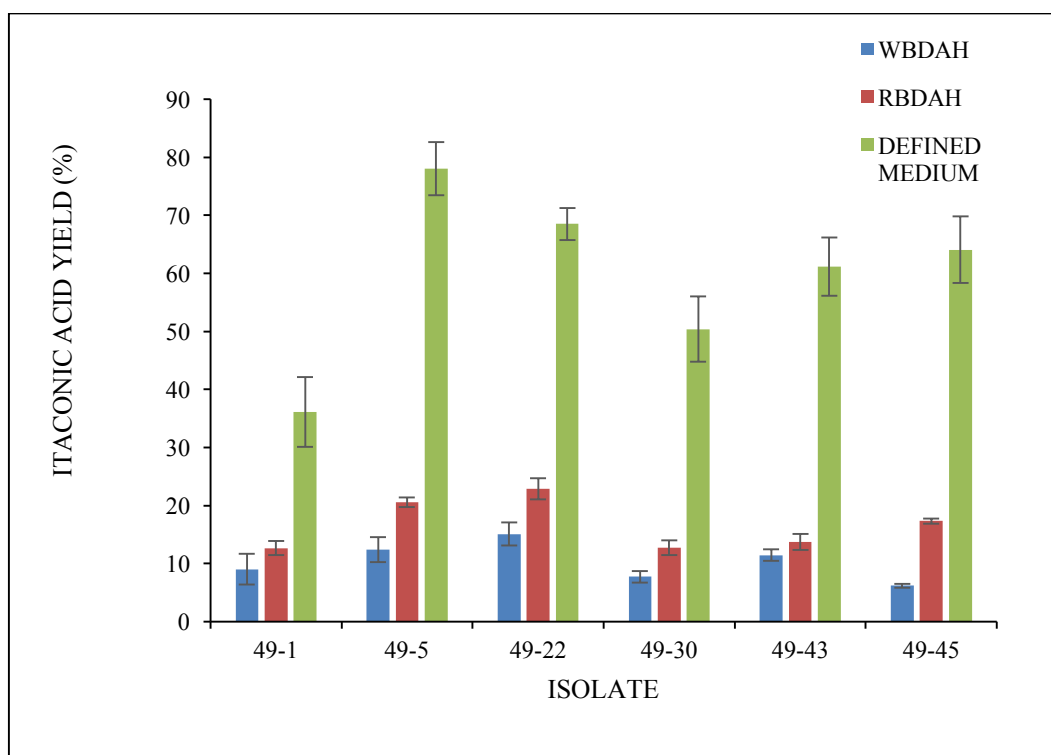


**Figure 4.23:** Time course of itaconic acid production during fermentation of sorghum dilute acid hydrolysates (DAH) bran or control glucose medium by *Aspergillus terreus* isolates 49-5, 49-22 and 49-45. The fermentation conditions employed were: 37 °C, agitation/aeration rate 200 rpm, pH 3.1. Left: Itaconic acid yields. Right: sugar consumption trend showing residual glucose over time. Values are averages of triplicate fermentations and error bars represent standard deviations.

Meanwhile, the control medium showed a different trend in that the fermentation proceeded without any decrease in itaconic acid levels even when production was halted at a later point of Day 7. The highest yielding strain was 49-22 with 24.7 g/l while the lowest yield of 12.1 g/l was from 49-45. Yields from 49-5 initially lagged, but by Day 5 surpassed that of 49-45 and was still increasing till the end of the experiment. Even despite the relatively high levels of residual glucose, fermentation was effectively halted by Day 2 in strain 49-45, while 49-22 and 49-5 appeared more robust producing 54.5 % and 40.4 % of the theoretical maximum itaconic acid respectively.

#### **4.3.2.3 Itaconic Acid Yields**

Having identified that maximum amounts of itaconic acid were produced at Day 4 when using RBDAH and WBDAH as the sorghum bran hydrolysate source, a further in-depth experiment was then performed to identify the best *A. terreus* isolates for use in this fermentation. The six highest-yielding isolates of *A. terreus* (49-1, 49-5, 49-22, 49-31, 49-43, 49-45) identified from the preliminary screen of 46 isolates were therefore used in fermentation of the hydrolysates, together with glucose control medium (Kuenz *et al.*, 2012). The results are presented as % of theoretical maximum yield as this gives an indication of efficiency of product formation and thus how economical the process is (**Figure 4.24**).



**Figure 4.24:** Itaconic acid yields of the best-performing isolates of *A. terreus* obtained from the screening experiment after fermentation in sorghum bran hydrolysates for 4 days, or on control defined glucose media for 7 days. Values are (%) of the theoretical maximum possible and are averages of biological triplicates. Error bars indicate standard deviations.

In contrast with the WBDHAH with a range of 6.2 % – 15.1 % and the RBDHAH with 12.7 % - 22.9 % itaconic acid yield, the glucose fermentations attained up to 78 % of the maximum possible yield (with isolate 49-5) indicating that the sorghum bran medium is not as optimally suited as the defined medium for itaconic acid production, at least under the conditions of these experiments. The % conversion rates corresponded to average actual yields of between 4.3 g/l and 7.8 g/l for the RBDHAH, 2.1 g/l and 5.2 g/l for the WBDHAH, and 25.0 g/l to 63.1 g/l for the defined medium (raw data not shown).

One way ANOVA showed itaconic acid yields (%) were significantly different based on medium type ( $F = 0.67$ ;  $df = 2$ ;  $P = 0.0003$ ) with multiple range tests showing differences between each pair of means. RBDHAH was the better hydrolysate supporting strain 49-22 to produce 22.9 % of maximum compared to 15.1 % obtained on WBDHAH. The performances of the strains were also found by one-way ANOVA to be significantly different on glucose medium ( $F = 25.17$ ;  $df = 5$ ;  $P = 0.0000$ ). However with WBDHAH and RBDHAH the data did not meet the assumptions underlying ANOVA. Therefore Kruskal-Wallis tests were used,

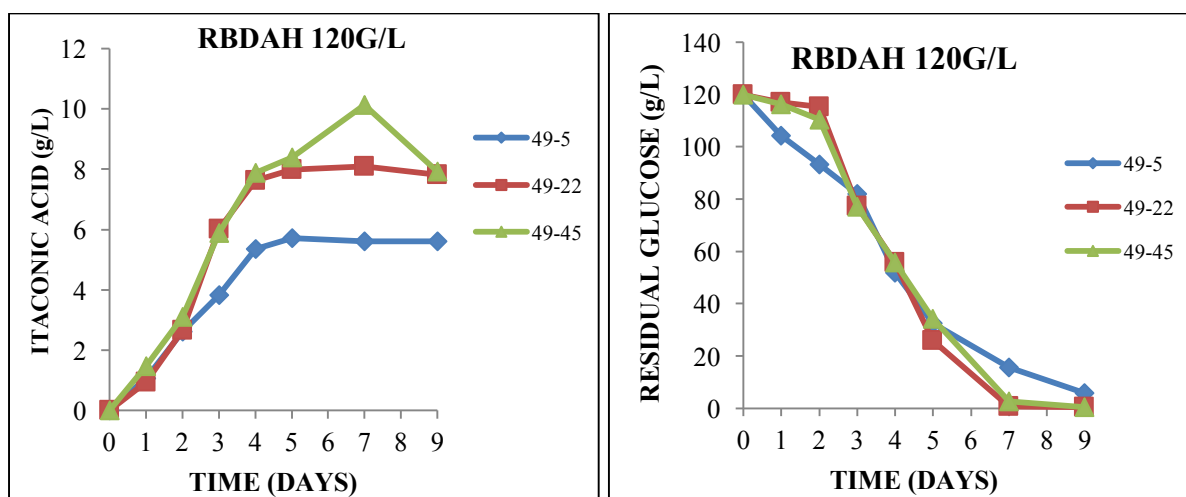
which showed that there were significant differences between the medians of yields [(H = 14.0292; df = 5; P = 0.0154) and (H = 14.834; df = 5 ; P= 0.0111) respectively]. A Type III Sums of Squares ANOVA for IA yield did not find any statistical significance in the interactions between medium and strain in the fermentations (F = 10.53; df = 10; P= 0.9988).

#### ***4.3.2.4 Effect of Glucose Levels on IA Production; Enriched RBDAH Fermentation***

##### **Enrichment Experiments**

Due to the observation from time-course experiments that the glucose in the hydrolysates was rapidly depleted, correlating with a decrease in IA levels, glucose enrichment of the DAHs was considered as a possible means to promote IA production. RBDAH was chosen as a representative test hydrolysate. The RBDAH was enriched up to 120 g/l using sterile concentrated glucose syrup [this value chosen to make it equivalent to the glucose concentration of the defined itaconic acid fermentation medium of (Kuenz *et al.*, 2012)] before fermentation was started. Media were then fermented under the same conditions as used previously, utilising the same three test isolates 49-5, 49-22 and 49-45 (**Section 4.3.2.2**).

A different outcome to that using RBDAH alone (**Figure 4.23**) was observed in that with the supplemented RBDAH media there was no decrease (i.e. removal) in IA levels after production peaked in two of the strains (49-5 and 49-22), while in 49-45 the itaconic acid yield only began to decline slightly after Day 7 when the glucose had been exhausted (**Figure 4.25**). However, critically the overall levels of IA yield remained only between 5-10 g/l even with the supplementation of extra glucose, so did not show a noticeable increase compared to use of RBDAH fermentation alone at 4 days. For example, the highest itaconic acid yield from 49-45 was at Day 7 at 10.1 g/l corresponding to only 10.2 % of the theoretical maximum. Meanwhile the maximum yields for 49-22 were almost exactly equal to those obtained with RBDAH fermentation alone. The glucose was exhausted by Day 7 in all isolates except 49-5 (**Figure 4.25**).



**Figure 4.25:** Time course of itaconic acid fermentation of glucose-enriched red sorghum bran dilute acid hydrolysate (RBDAH) (starting concentration of 120 g/l), by *Aspergillus terreus* at 37° C and 200 rpm for 9 days Left: Itaconic acid yields; Right: sugar consumption trend showing residual glucose over time.

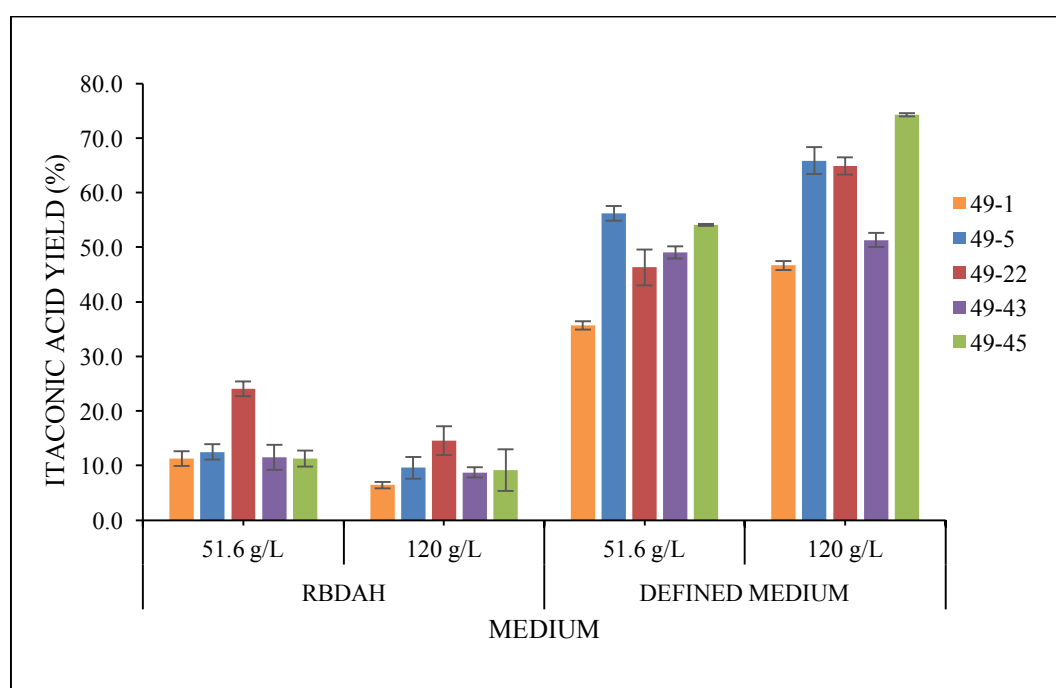
### Effect of Glucose Levels

Given the observation that supplementation of the RBDAH with glucose did not markedly increase IA production, (**Figure 4.25**) an experiment was performed to determine the production of IA when using the defined media of (Kuenz *et al.*, 2012) with a similarly lower glucose content as that of the RBDAH media. It was anticipated that this might reveal whether differences in IA yield were due simply to differences in the glucose levels of the respective media, or whether some other chemical components of the media were also responsible for the differences in yield. The RBDAH was terminated after 4 days and all others after 7 days and samples analysed.

It was observed that decreasing the glucose content of the defined media to that comparable to the native RBDAH media (51.6 g/l) resulted in a decrease in IA yield in most of the five *A. terreus* isolates assayed (compared to the original 120 g/l level) (**Figure 4.26**). However, a key result was the IA yield of all the isolates remained noticeably higher with the defined media containing glucose even at 51.6 g/l compared to the RBDAH medium. This indicated that the defined media of (Kuenz *et al.*, 2012) is very well suited for itaconic acid production and/or that the RBDAH media contained some possible inhibitory compounds. It was also observed that for the defined media, the higher glucose concentration of 120 g/l was more productive. For example, IA yield by 49-45 rose over 20 % when glucose levels were increased. However, the increase in production efficiency was not proportional as the richer

medium contained more than double the glucose content as the poorer medium, whereas IA outputs only improved marginally for the glucose consumed. Results also confirmed that supplementation of the RBDAH media with additional glucose did not result in marked increase in IA production (**Figure 4.26**).

The results did not meet the assumptions underlying the use of a one-way ANOVA, so a Kruskal-Wallis test was used to statistically analyse the difference in the median values of itaconic acid by medium. There was no significant difference in IA yield among the strains with respect to specific media ( $H = 0.008$ ;  $df = 1$ ;  $P\text{-Value} = 0.929$ ). However, yields between media were significantly different ( $H = 44.2623$ ;  $df = 1$ ;  $P\text{-Value} = 0.0000$ ), confirming that the defined media performed significantly better than the RBDAH even at similar glucose contents.

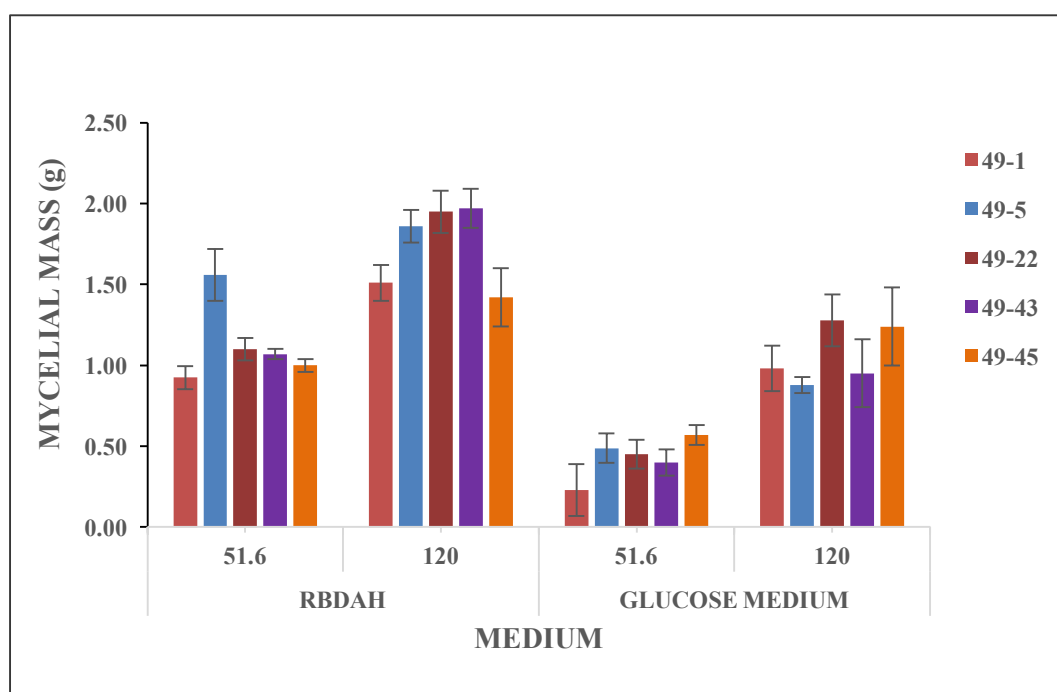


**Figure 4.26:** Itaconic acid yields from the fermentation of red bran hydrolysates and defined medium at 37 °C and 200 rpm. Yields are the (%) of the theoretical maximum possible and are average of triplicate experiments. Error bars indicate standard deviations. Values below the x axis indicate the media glucose content (note that the native RBDAH contains 51.6 g/l glucose without supplementation).



## Mycelial Mass

Finally, the end point mycelial mass was determined to see if there was any correlation between mycelial mass produced in the various fermentations and IA production. Findings are presented in **Figure 4.27**. Intriguingly, the final mass was found to be significantly higher when using RBDAH as a growth substrate than the defined medium at both 51.6 and 120 g/l glucose levels ( $F = 1046.64$ ;  $df = 1$ ;  $P = 0.0000$ ). Importantly, this demonstrated that the RBDAH did not inhibit mycelial growth relative to the defined media, indeed RBDAH significantly enhanced growth.



**Figure 4.27:** Mycelial mass produced by the *Aspergillus terreus* isolates in fermentation on red bran dilute acid hydrolysate (RBDAH) and defined medium at two different concentrations. The experiments were terminated on Day 7 except for the 51.6 g/l RBDAH, which was terminated on Day 4. Values presented are averages of biological triplicates. Error bars represent standard deviations.

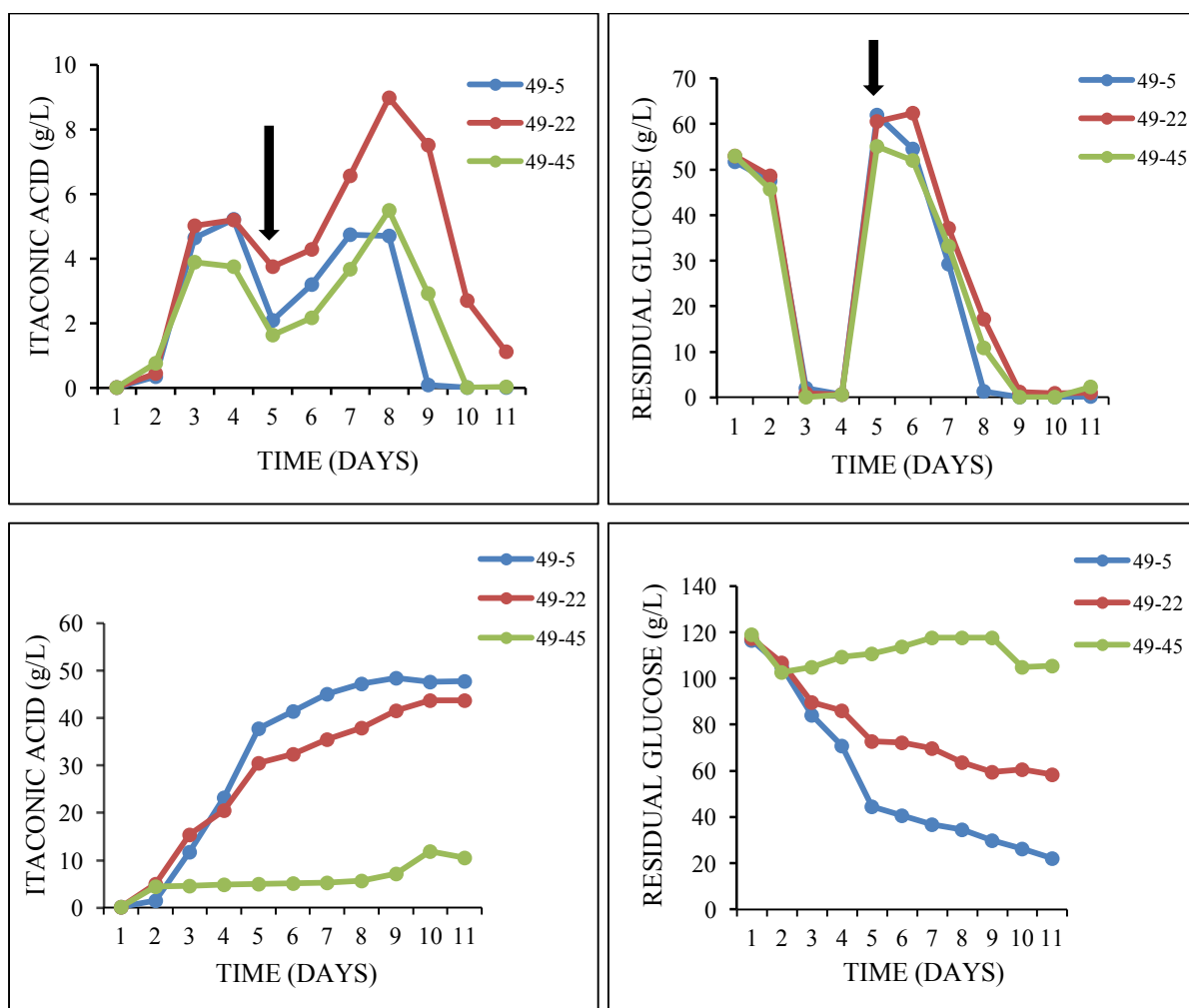
### 4.3.2.5 Fed-Batch Fermentations

In a further attempt to increase IA production from RBDAH the impact of glucose replacement on itaconic acid yield was investigated in a fed-batch fermentation. It had previously been observed that itaconic acid yields (g/l) on RBDAH begin to decline after Day 4 correlating with exhaustion of glucose levels (**Figure 4.23**). Therefore it was considered

possible that supplementing the RBDAH media at this point so that glucose levels were raised on Day 5 might raise IA production to levels more similar to those in the glucose control fermentation.

Fermentations were monitored over a 11 day period, and compared to production from the standard glucose defined media (**Figure 4.28**). All isolates of *A. terreus* showed broadly similar outcomes. Results concerning IA production on RBDAH were similar to previous data (**Figure 4.23**) in that a peak in IA levels was observed up to Day 5 when the glucose in the medium had been exhausted and then the IA levels began to decline. However, glucose replenishment at 55 g/l – 60 g/l at Day 5 then resulted in a resumption of IA production, with a further peak in production between Days 7 and 8 according to particular isolate before the glucose was again completely exhausted by Day 9 (**Figure 4.28**). For example, isolate 49-22 produced 5.2 g/l IA on Day 4, which rose to 9.0 g/l after replenishment on Day 5. However, critically the levels of IA production even with the fed-batch supplementation of glucose, remained relatively low at ca. 4-9 g/l IA compared to the standard glucose media which yielded approximately between 10-50 g/l depending on the isolate (**Figure 4.28**). Thus, this approach did not offer any major advantage.

A Kruskal-Wallis test was performed to statistically analyse the production of itaconic acid by strain as the values failed Levene's variance test. There were no significant differences in IA yields between the strains across both media ( $H= 4.3830$ ;  $P= 0.1117$ ). A multifactor ANOVA to determine the individual effects of strain and medium type, and their interaction on IA output showed significant contributions ( $P= 0.0001$ ;  $0.0000$  and  $0.0002$  respectively). One-Way ANOVA of each medium confirmed that there was a significant difference between means of yields by each isolate in defined medium, but not in RBDAH. A multiple range test further revealed the differences between the yields of the strains and shows that 49-5 and 49-22 have similar yields (**Table 4.3**).



**Figure 4.28:** Fed-batch fermentation of red bran dilute acid hydrolysate (RBDAH) with three *A. terreus* isolates over 11 days. Top Left: Itaconic acid production in RBDAH; Top Right: Glucose consumption in RBDAH fermentation; Bottom Left: Itaconic acid production in glucose defined medium; Bottom Right: Glucose consumption in defined medium. Arrows indicate point of glucose replenishment.

**Table 4.3:** Multiple Range Tests for itaconic acid by strain

Contrast	Sig.	Difference	+/- Limits
49-22 - 49-45	*	11.955	9.10693
49-22 - 49-5		-1.14273	9.10693
49-45 - 49-5	*	-13.0977	9.10693

\* denotes a statistically significant difference.

## 4.4 Discussion

Sorghum bran is a potentially useful feedstock for a wide range of biotechnological applications. Work in this chapter investigated the potential of various hydrolysates made from sorghum bran to produce ethanol and itaconic acid via fungal fermentations. Four hydrolysates, white bran enzyme hydrolysate (WBEH), white bran dilute acid hydrolysate (WBDAH), red bran enzyme hydrolysate (RBEH) and red bran dilute acid hydrolysate (RBDAH) were evaluated for their ability to support the growth of various yeasts and *Aspergillus terreus*. This was achieved using initial screening tests to identify the best strains and media, which were then employed in subsequent optimisation experiments.

### 4.4.1 Ethanol Production

Firstly, the potential for several yeast species to produce ethanol on the various sorghum bran hydrolysates was investigated.

#### 4.4.1.1 Screening Tests

The yeast species, and strains thereof, employed in this study were selected based on various physiological characteristics they were reported to possess ranging from high ethanol yields (*Saccharomyces*) (National Collection of Yeast Cultures, 2016) to the ability to ferment pentose sugars (*Candida* and *Scheffersomyces* strains) (National Collection of Yeast Cultures, 2016). Screening tests were initially performed which aimed to determine which strains were best suited to the conditions provided by the hydrolysates, and to identify any differences in the performance of the hydrolysates. Ideally more strains of each yeast species would have been included to ensure representation of the genetic diversity of the species, but some compromises had to be made due to limited time available.

A spot plate test was performed using hydrolysate agar plates and it was observed (**Figure 4.14**) that all the yeasts successfully metabolised and grew on the different media under both aerobic and anaerobic conditions. Regarding the effect of hydrolysate type, the colonies resulting from all the dilutions plated on the dilute acid hydrolysate (DAH) medium were smaller than those on their enzyme hydrolysate (EH) equivalents. The strains grew less vigorously on the DAH plates, which was considered most likely due to the lower glucose

content, which was about 33.6 g/l in this batch as compared to about 54 g/l in the EH. Notwithstanding, the 10- and 100-fold dilutions of every strain still grew vigorously on these plates, this being particularly true for *W. anomalous*. The higher dilutions showed less growth on both media, as was to be expected due to the smaller inoculum size, but in yeasts such as *C. arabinofementas*, *S. cerevisiae* NCYC 2592 and *W. anomalous* this was not obvious.

The aerobic plates had more defined growth while the colonies on the anaerobically incubated EH plates showed mat-forming, spreading tendencies across every dilution. Yeast species show remarkably complex, strain-specific morphologies that are influenced by the environmental conditions including showing different morphologies under different conditions in the same media, and in different media under the same conditions (Voordeckers *et al.*, 2012). For example, the gene *FLO11* encodes a large cell-surface protein which is crucial for colony development and morphology and the formation of pseudohyphae (chains of elongated cells at the edge of the colony) under nutrient deficient conditions. *FLO11* is also involved in the formation of large thin biofilm-like colony structures called “mats” on semi-solid media (Voordeckers *et al.*, 2012) and it is possible that the absence of oxygen in anaerobically incubated plates as seen in **Figure 4.14** (A and C) triggered this growth phenotype in a similar way.

In the Phenotypic Microarray (PM) analysis, changes in colour of a redox sensitive dye were monitored during respiration in the selected medium in a 96-well plate (Bochner *et al.*, 2001). This colour change measured the yeast’s ability to utilise the sugars in the medium and thus was an indicator of metabolic activity. With the PM analysis, all the strains showed typical growth curves except for *W. anomalous* which had a relatively sharp decline in redox signal intensity at 10 h in all the media, before plateauing into the lag phase (**Figure 4.15**). The variation in the signal intensity was considerable, ranging from 65.7 for *S. stipitis* to 116 for *S. pastorianus* at 70 h on RBEH and 58.7 – 106.7 for *S. stipitis* and *S. cerevisiae* 1119, respectively, on WBEH. A similar trend was observed with the DAHs where the pattern of the growth signal was almost exactly the same for all species on both bran hydrolysates, except for *S. cerevisiae* 1383. This strain is a genetically characterised strain which can only utilise glucose semi-anaerobically (National Collection of Yeast Cultures, 2016) in which case the observed growth might even be considered impressive. In the hydrolysates *S. stipitis* showed least metabolic activity while *C. arabinofementas* and *S. cerevisiae* 1119 showed the most activity. *S. cerevisiae* 1119 is an ale production strain (National Collection of Yeast Cultures, 2016) which accounts for the high performance and similarity in profile with

NCYC 2592. The reason for the poor growth of *S. stipitis* was not clear, particularly as it can utilise pentoses and is a Crabtree-negative organism i.e. it is able to respire (and produce biomass) under aerobic conditions regardless of sugar concentrations (Deken 1965). Low redox signal intensity of *S. stipitis* in PM analysis using glucose as sole carbon source was also reported on macroalgae hydrolysates and wheat straw hydrolysate (Pensupa, 2015; Kostas *et al.*, 2016).

Generally, the redox signal intensities of incubation on EHs were about double that of DAHs, which was directly correlated with the concentration of sugars. Metabolic activity was relatively similar among all strains and between hydrolysates of same type (based on method of preparation), except for *S. cerevisiae* 1383 which exhibited a longer lag phase. The similarity in the metabolic profiles of strains on both bran types indicated that either WB or RB could adequately support the growth of the yeasts, and that both EH or DAH provided adequate nutrient source for growth.

#### **4.4.1.2 Mini-Fermentations**

Next, mini-fermentations were performed to determine if the yeasts were actually able to ferment sugars in the medium to ethanol, given that the PM assays only gave an indication of overall metabolic activity. Also, because the RBDAH is coloured red, there was a concern that the PM analysis for this medium may not have been reliable as the colour may have interfered with the detection of the tetrazolium dye.

In the mini-fermentations a steady decline in weights of the vessels was observed which could be detected after about 2 h of incubation, correlated with the catabolism of monosaccharides into CO<sub>2</sub>. The red bran hydrolysates showed higher weight loss than the white bran and this indicates that the yeasts were able to more vigorously ferment the latter. The enzyme hydrolysates had a higher glucose content than the acid hydrolysates, which is thought to explain the higher weight loss observed in the former due to the greater potential for fermentation and CO<sub>2</sub> release. RBEH was the best medium in terms of weight loss, losing 2.2% of the vessel weight compared to WBEH with 1.4 % while YPD only showed a loss of 1.0 %. This is higher than previous reports of the highest weight loss of 0.25 % - 0.35 % by yeasts on hydrolysates of two macroalgae *Chondrus crispus* and *Palmaria palmata* (Kostas *et al.*, 2016).

There were differences in the progression of fermentation among strains although the overall trends were similar. The weight loss shown by *S. cerevisiae* NCYC 2592 in all media was almost complete after 48 h whereupon the culture entered the stationary phase, while *S. pastorianus* had the longest lag phase and least weight loss (**Figure 4.16**). *S. cerevisiae* NCYC 2592 also showed the highest weight loss in all media except RBDH, in which *K. marxianus* showed maximum weight loss. This was expected as strains of *S. cerevisiae* are the preferred organisms for bioethanol production due to their ability to easily utilise hexose sugars and their high osmotic and ethanol toxicity tolerance (Zhao and Bai 2009). Indeed, strain NCYC 2592 is described on the National Collection of Yeast Cultures (NCYC) website as a distiller's production strain with the ability to aerobically and semi-anaerobically utilise glucose and many other sugars with a good ethanol yield.

#### **4.4.1.3 Ethanol Yields**

The fermentations were terminated and analysed for ethanol production. A key result was that all of the yeasts were shown to produce ethanol from all types of the sorghum bran hydrolysates, in concentrations ranging from (**Table 4.2**). The highest yields of 24.4 g/L was obtained by *K. marxianus* fermentation of RBEH. This corresponded to 88.9 % conversion of the glucose consumed into ethanol. The final concentrations were found to be high and corresponded to high conversion of glucose ranging from approximately 60.1 % - 88.9% (**Figure 4.17**). This was significant as these results show that sorghum bran hydrolysates can be used for bioethanol production. Although there was no statistically significant difference between ethanol yields of the yeast strains, the best performing was *K. marxianus* which is impressive considering that it is a Crabtree-negative organism. Though not as widely used in the biotechnology industry as *S. cerevisiae*, this non-conventional yeast is attracting increasing attention as it can utilise various substrates including xylose, lactose and inulin to produce ethanol, which might explain its superior performance to *S. cerevisiae* in the present fermentations (Gao *et al.*, 2015).

It was observed that small amounts of acetic acid were also produced, so this was also estimated and presented in **Figure 4.17**. Small amounts of acetic acid could favourably impact the ethanol yields as it has been suggested that acetic acid improves transportation capacity across the plasma membrane which could potentially affect sugar uptake rate leading to increased ethanol formation (Taherzadeh *et al.*, 1997). However, higher amounts

have been shown to inhibit ethanol formation (Nigam, 2001) and leads to synergistic effects with other inhibitors, further suppressing ethanol formation (Greetham *et al.*, 2014).

The best medium for ethanol production was the RBEH, with up to 89 % conversion of glucose into ethanol when using *K. marxianus*. Considering that 5-12 % of carbohydrate is usually converted to cells this means usually less than 0.47 g/g conversion of carbohydrate to ethanol occurs (Lynd, 1996) as against the 0.51 stoichiometric theoretical maximum. This means realistically only a maximum of 92.1% of the theoretical maximum is actually possible in practice because some carbohydrate is required for cellular materials. This makes the yield of 89 % by *K. marxianus* particularly impressive. Performance on RBEH was higher than was observed in YPD while WBDAH showed the lowest conversion rates. However, the RBDAH still attained over 70 % conversion rates with *K. marxianus* and *S. pastorianus*, and therefore this medium was selected for subsequent fermentations as it is much economically cheaper to produce hydrolysates by thermochemical methods than by using enzymes.

#### **4.4.1.4 Time Course Fermentation**

The best performing strain, *K. marxianus* was fermented solely on RBDAH (which contained 45 g/l glucose) and the time course determined. Fermentation plateaued after 72 hr (**Figure 4.18**), producing 7 g/l ethanol corresponding to 63.5 % of the theoretical maximum possible from the 23.7 g of glucose consumed. It is unclear why the fermentation did not proceed to completion in this case, and it was not immediately possible to investigate the possible causes, however it might have been due to the exhaustion of limiting nutrients such as nitrogen source, resulting in high unused sugar content. Viable cell counts were performed at each time point and it was observed that the decline phase commenced after 72 hr, which coincided with the point at which the ethanol fermentation also stopped (**Figure 4.19**). This might also be explained as likely due to a depletion of key nutrients such as N or P.

This yield is lower than was obtained from the previous experiment, but the efficiency of conversion was still relatively high indicating minimum glucose wastage by the yeast which can be considered fair considering that ethanol yields obtained from fermentation of dilute acid hydrolysates are usually between 50 – 60 % of the theoretical values (Wyman, 1994). This is higher than 2.1 g/l obtained from whey by same organism (Zafar and Owais, 2006). However, it is in agreement with yields of 61 % - 68 % of the theoretical maximum obtained



from *K. marxianus* CECT 10875 fermentation of hydrolysates of various lignocellulosic materials (Ballesteros *et al.*, 2004). It is also similar to 63 % - 84 % of theoretical maximum reported by Stenberg and co-workers in simultaneous saccharification and fermentation of softwood (Stenberg *et al.*, 2000); and to the 85 % of theoretical maximum by the simultaneous saccharification, filtration and fermentation of spruce chips (Ishola *et al.*, 2013). Thus, the results indicate a possible applied potential to use sorghum bran hydrolysates for economical production of bioethanol (see concluding remarks in **Chapter 7**).

#### **4.4.2 Itaconic Acid Production**

Secondly, the potential for *Aspergillus terreus* to produce itaconic acid (IA) when using the various sorghum bran hydrolysates was investigated.

##### **4.4.2.1 Screening of *Aspergillus terreus* Isolates**

A selection of isolates of *A. terreus* were fermented on a defined glucose medium which was previously shown to promote the production of IA under in vitro conditions (Kuenz *et al.*, 2012). In accordance with this previous report, IA production was indeed observed in the majority of isolates assayed (35 out of 46 isolates; 76%). However, there was significant variation in the yields with most isolates were being relatively low producers with few high-producers (**Figure 4.21**). The right-skew of the histogram (**Figure 4.22**) shows that IA production in field *A. terreus* isolates is not normally distributed. This deduction was arrived at because even though this sample size was admittedly small ( $n = 46$ ), the collection of isolates came from a wide range of clinical and environmental sources (**Appendix 1**).

Production of secondary metabolites by fungi has been reported to follow a non-normal distribution, often requiring transformation to obtain Gaussian distributions. For instance, DON-production by *Fusarium* spp. in wheat were found to generally be in low quantities and to follow a log-normal distribution (Klix, 2007). This might be because the metabolite is not essential to growth and might only be of selective advantage under rare environmental conditions, so that many isolates might survive without production or by producing just low quantities. In the case of *A. terreus* it is likely that IA is produced as a secondary metabolite in order to lower the pH of its environment and provide a selective advantage over other competing microorganisms which might not be able to withstand the acid conditions.

The highest producer was isolate 49-43 (from a clinical source from Athens, Greece; **Appendix 1**); others were 49-5 and 49-22 from environmental sources thus showing the value of screening for natural field variation amongst fungi. Representatives of these high producing isolates were then selected for further research involving fermentation of the sorghum bran hydrollysates.

#### **4.4.2.2 Time Point Fermentations**

The time point fermentations showed that itaconic acid can be produced from sorghum bran, a key finding with respect to possible exploitation of sorghum bran waste to produce value-added products. On the control glucose medium adapted from Kuenz et al. (2012) yields of IA increased during the time course of fermentation before reaching a final plateau. However, a different pattern was evident when using sorghum bran as the feedstock, with some further variation evident between isolates. Itaconic acid was produced after a brief lag phase of 2 days in RBDAH and 1 day in WBDAH with yields rising until Day 4 for 49-22 and 49-45 in both media, but thereafter a marked fall in levels of IA occurred (**Figure 4.23**). With 49-5, production started and stopped sooner, which meant there was a shorter lag period for this strain. There are two likely reasons for this phenomenon of IA decline. First, it is likely that the IA produced was being converted into another end product such as pyruvate or most likely, itatartaric acid. While it was not possible to demonstrate the presence of itatartaric acid in these experiments, and visual observations of the chromatograms obtained in the later days of fermentation did not show an increase in any other metabolites in the profile, these do not conclusively eliminate this explanation. The second possibility is that as a result of glucose depletion, the *A. terreus* isolates began to catabolise the itaconic acid to utilise it as a carbon-source. This hypothesis is based on a recent report that showed that *A. terreus* can catabolize itaconic acid in three successive steps: first into itaconyl-CoA by itaconyl-CoA transferase with succinyl-CoA as the CoA donor, then itaconyl-CoA is hydrated by itaconyl-CoA hydratase into citramalyl-CoA which is then finally cleaved into acetyl-CoA and pyruvate by citramalyl-CoA lyase (Chen *et al.*, 2016). This second theory is interesting as the point of itaconic acid removal was exactly the same as point of glucose exhaustion i.e. around Day 4. Also, the only strain with some residual itaconic acid by Day 9, 49-22 was the one in which the broth contained residual glucose for the longest time. This is a phenomenon observed in the diauxic growth of *S. cerevisiae* in aerobic conditions where ethanol and biomass are formed in the presence of excess glucose, and then upon glucose depletion growth continues

with ethanol as substrate after a lag phase (Nielsen et al., 2012). It was thus decided that subsequent fermentations with sorghum bran hydrollysates would be terminated on Day 4.

In both RB and WB media, 49-22 was the best-performing strain, with highest production (5.5 g/l) on RBDAH. As control for the RBDAH and WBDAH, defined glucose-based medium (**Section 2.1.4**) was also fermented under the same conditions. As evident in **Figure 4.23**, isolates 49-5 and 49-22 showed a continual increase in IA production up to Days 7-9, whereas strain 49-45 almost ceased IA production after the third day on the defined media which could be due to an unidentified problem with the fermentation. Despite the fact that isolate 49-45 (NRRL 1960) is one of the most widely reported *A. terreus* strains used for itaconic acid production in the literature (**Table 4.1**), it nevertheless appeared that the 'in-house' isolate 49-22 was the most robust strain with peak IA production of 25 g/l on the defined medium (by Day 7) which was over 0.39 g/g of itaconic acid produced per gram glucose consumed (or 54.5 % of maximum conversion). The overall yields obtained in the present work were lower than those reported elsewhere of 65 g/L after 10 days fermentation with the same medium (Kuenz et al, 2012). Higher yields of up to 91 g/L were obtained with higher initial glucose contents of 180 g/L as they observed that higher glucose amounts eliminated substrated limitation and the fermentation was in a stirred tank reactor (not shake flasks) and may explain why they had better overall performance compared to the original medium with 120 g/L.

The lower yields of the time point fermentations (about 25 g/l) may also be been due to a disruption in IA production caused by the periodic sampling, and were lower than those obtained in the initial screening of about 34 g/l (for 49-22). Glucose utilisation was relatively similar in all three isolates in this defined medium.

#### **4.4.2.3 Itaconic Acid Yields**

Having established that the ideal length of fermentation for optimum production of IA was for 4 days on both sorghum bran media and 7 days on the defined glucose medium, further replicated fermentations were then set up under these conditions and the yields of representative isolates on the different media compared. Itaconic acid values are presented as yields (%) of the theoretical maximum possible (**Section 4.2.2.3**) in order to compare productivities more objectively on the basis of carbon (glucose) consumed. It was observed that similar to the time point experiments where the itaconic acid production in g/g was

highest in RBDAH and with strain 49-22, the same result was obtained here with yields as a function of theoretical maximum possible based on % glucose consumed (**Figure 4.24**). The RBDAH was significantly the better hydrolysate, while the difference in yields between *A. terreus* isolates was statistically significant. This is probably due to inherent variability at a genetic level associated with the wide range of sources from which the organisms were isolated (**Appendix 1**).

Itaconic acid yields on the sorghum bran hydrolysates were considered to be low even after accounting for carbon loss due to biomass accumulation and cell maintenance, as the hydrolysates all produced below 35 % of the theoretical maximum conversion. The reason for this is not exactly clear, but it could be due to the exhaustion of glucose in the medium for mycelia formation in the trophophase before secondary metabolite production had been properly established, thus leaving none for conversion to itaconic acid. However, the early start of itaconic acid production (**Figure 4.23**) even before visible mycelial accumulation (by Day 1 – Day 2) shows that the IA production process is not entirely uncoupled from the growth process, which indicates that the fermentation conditions might just favour lower itaconic acid production. By contrast, yields on the defined glucose medium were quite impressive, up to 78 % with 49-5 which proved the assumption that its previous low yields (of 40.4 %) was due to the consistent disruption of the sampling process. Similarly, 49-22 and 49-45 achieved 69 % and 65 % conversion rates, respectively.

Meanwhile, yield in terms of g/l IA varied between 4.3 g/l and 7.8 g/l for the RBDAH, 2.1 g/l and 5.2 g/l for the WBDAH, and 25.0 g/l to 63.1 g/l for the defined medium. These figures are lower than itaconic acid yields of 5.8 g/l upwards reported from a range of different feedstocks (Ahmed El-Imam and Du, 2014) (**Table 4.1**), although it was not possible in all of these reports to determine the yield % of theoretical maximum as the amounts of glucose consumed were not always available.

#### **4.4.2.4 Enriched RBDAH Fermentation**

The findings of **Section 4.3.2.2** showed that a decrease in itaconic acid levels (removal) on RBDAH coincided with glucose depletion, suggesting that IA produced up to that point was then being utilised as a carbon source or converted into other metabolites. It was speculated that a higher glucose content might allow the fermentation to proceed longer and provide higher itaconic acid yields. The RBDAH was therefore enriched to a similar glucose content

as the defined fermentation medium, while a defined medium with lower glucose content was also prepared. Both sets of media were fermented with daily sampling.

It was observed that the glucose-enriched RBDAH did not show a reduction in IA levels with time (**Figure 4.25**), which appeared to represent an improvement on use of RBDAH alone. However, IA production remained in the range 5-10 g/l i.e. relatively low yields. As a specific example, the yield of 8.8 g/l observed with 49-22 on RBDAH (**Appendix 3**) is higher than the value of 3.3 g/l reported for IA production from sorghum starch acid hydrolysates (Petruccioli *et al.*, 1999). The latter authors suggested that high phosphorus content of sorghum could account for the relatively low yields as it can negatively impact IA production.

In terms of conversion of glucose present, the yield of isolate 49-22 dropped to 14.6 % in the enriched RBDAH compared to 24.0 % in RBDAH alone (**Figure 4.26**). This was because although there was an increase in itaconic acid production (just over 2 g), it was at a cost of a disproportionately higher amount of glucose consumed (over 50 g), which makes the increase less than commensurate (**Appendix 3**). The fact that yields remained low in enriched media indicates that the reports that high initial glucose concentrations are required for high itaconic acid yields (**Section 1.2.8.5**) (Kuenz *et al.*, 2012, Willke and Vorlop, 2001) might apply specifically to the use of defined synthetic media and not be relevant to complex biomass hydrolysates.

A comparison of mycelia production at the end of the fermentation showed that RBDAH supported heavy biomass formation (**Figure 4.27**), even surpassing that of the defined glucose medium of Kuenz *et al.* (2012). Indeed, overall the highest biomass was achieved in the enriched RBDAH medium (being 4 – 5 times higher than in the low-glucose defined medium). These observations were critical, as they revealed that the lower IA production seen in the sorghum bran extracts was not due to inhibition of growth by certain factors in the dilute acid bran extract, indeed the reverse – the RBDAH promoted mycelial growth above levels seen when using the glucose defined medium of Kuenz *et al.* (2012). The RBDAH is a crude medium which is rich in nutrients including phosphorus (as discussed above and enumerated in **Section 3.3.1**). Thus, it will naturally support mycelial growth. Given that the defined medium of Kuenz *et al.* (2012) has been formulated and optimised to maximise itaconic acid production, this suggests that some nutrient stress might favour secondary metabolism and IA production. Therefore the considerable diversity of nutrients, including

glucose, in RBDAH leading to considerable microbial biomass may thus be responsible for the lower IA productivity observed.

#### **4.4.2.5 Fed-Batch Fermentation**

It was considered likely that the majority of phosphorus (and other nutrients) in the sorghum bran hydrolysate, that might have been inhibiting itaconic acid production, would have been taken up for growth during the trophophase. It was therefore postulated that addition of glucose at the start of the idiophase might sustain and lead to increased IA production in *A. terreus*, should the glucose be used as a carbon supply for secondary metabolism. Therefore, additional glucose was introduced on Day 5 to the RBDAH fermentations (to a final concentration of about 60 g/l). Although this led to further IA production, the increase in IA yield was not considerable (**Figure 4.28**). Itaconic acid production resumed upon glucose replenishment up to around 5.5 g/l for 49-5 and 49-45 and 9.0 g/l for isolate 49-22 by Day 8, before a major fall in IA levels in all fermentations.

Fed-batch fermentations are intermediate between batch and continuous culture in that they can be more efficient than batch fermentations, which use up a good portion of the nutrients for biomass formation leaving limited nutrients and time period for metabolite production, while also requiring less medium than continuous culture (Hunter, 2007). Fed-batch fermentations have not been widely applied to itaconic acid production as no reports were found. In the current study the fed-batch approach showed similar overall result, in terms of IA yield, to those results obtained by enrichment of the hydrolysate, achieving a yield of 9 g/l. IA (equivalent to 11.3 % of the theoretical maximum). This indicates that glucose addition can increase the total amount of IA produced, but will reduce the yield per gram of glucose consumed as the fermenting organism used most of the glucose for cell maintenance.

Thus overall, the various IA fermentation strategies increased yields in terms of g/l but the productivity based on the efficiency of conversion of glucose utilised was not very high. Given that this factor is of paramount importance in order to make itaconic acid production from sorghum bran economically competitive, results therefore indicated that the use of sorghum bran hydrolysates in their native forms for production of IA with wild-type isolates of *A. terreus* might not be commercially viable. It was thus apparent that the application of methods to improve the yields obtainable from the microorganism-medium system were necessary.

## 4.5 Summary and Conclusions

In this chapter the use of four sorghum bran hydrolysates to produce the value-added products ethanol and itaconic acid was investigated.

- Screening tests revealed that the hydrolysates supported yeast growth and metabolism with *S. cerevisiae* NCYC 2592, *S. pastorianus* and *K. marxianus* standing out.
- Weight loss of over 2 % were observed in mini-fermentations dependent on yeast strain and media type. Ethanol yields of 71 - 89 % were observed in RB hydrolysates and 63 - 80 % with the WB hydrolysates. *K. marxianus* had the overall highest levels of ethanol production, although differences between strains were not significant.
- There were significant differences between the hydrolysates, with better production obtained from enzyme hydrolysates than dilute acid hydrolysates, none based on strain.
- Fermentation using RBDAH with *K. marxianus* yielded 7 g/l ethanol, corresponding to 63.5 % glucose conversion of the theoretical maximum and this was due to an apparent truncation of the fermentation with half of the glucose not consumed. Even though the previous results show that it is possible to achieve high yields and concentrations with this strain on sorghum bran hydrolysates, further work needs to be done with this strain.
- 46 isolates of *A. terreus* screened showed the ability to produce itaconic acid. There was not a normal distribution, with a skew towards most isolates being low- or non-producers.
- Time-point fermentation with the representative strains on sorghum bran hydrolysates showed that the DAHs were able to support itaconic acid production. However, IA production could not be reliably replicated using EHs.
- IA production peaked at Day 4 in DAH fermentations. Highest levels of IA were achieved using isolate 49-22 and RBDAH medium.
- Due to low conversion yields (about 30 % of maximum possible), glucose enrichment of the RBDAH and fed-batch fermentations were investigated and were found to increase output (in g/l) but decrease productivity per unit glucose consumed (% of maximum).

The aims of this chapter have been met as sorghum bran hydrolysates have been successfully utilised for biotechnological conversions. It was shown that a medium that was cheap and easy to prepare from it, RBDAH, can support yeast and *A. terreus* growth and was successfully applied for the production of a value-added fuel and a chemical, bioethanol and itaconic acid, in yields which were modest but still comparable with the amounts in literature.

## Chapter 5: Hydrolysate Purification

From experimental work undertaken in **Chapter 4**, it was evident that the sorghum bran hydrolysates were able to support the growth of various yeasts and *Aspergillus terreus* to produce ethanol and itaconic acid, respectively. However, while ethanol concentration (g/l) and productivity based on glucose consumption were high, they were comparatively low for itaconic acid. Due to the low yields of itaconic acid observed in **Chapter 4**, it was decided that the effect of purification of the red bran sorghum hydrolysates on itaconic acid production would be investigated.

### 5.1 Introduction

Several processes exist for the production of biomass hydrolysates which can then be used for the downstream production of value-added products and these have been discussed in **Section 1.2.7**. However, these processes can generate several compounds which are inhibitory to the intended downstream process. For example, these hydrolysates often contain, in addition to the various sugars produced during hydrolysis, a range of toxic compounds typically generated during the pretreatment and hydrolysis processes (**Section 3.1.3**). They may also contain several anti-microbial compounds originally present in the biomass which pass into solution. These substances are generally termed inhibitors (usually of ethanol fermentation) and have been discussed briefly in **Section 3.1.3**.

Cellulose and hemicellulose form the main carbohydrates in lignocellulosic biomass, and in sorghum bran, like other cereal brans, relatively high amounts of starch are also found with 16.4 - 33.2 %, % and reported for wheat bran, 51.0 % for barley bran and 52.3 % for oats bran respectively (Clegg, 1956; Englyst *et al.*, 1983; Bhatta, 1993). The pretreatment of this carbohydrate to convert it into fermentable sugars is a necessity in bioconversion processes. The non-specificity of the various pretreatment processes, particularly the acid pretreatments result in the formation of substances that are potentially inhibitory to microbial processes. High-temperature and pressure pretreatment of biomass tends to result in limited degradation of hexoses, which results in the production of only small quantities of HMF thereby mitigating the effect. On the other hand, similar pretreatment of hemicellulose results in



increased production of HMF, in addition to furfural and low molecular weight acids such as levulinic acid (Chandel et al., 2011).

Phenolic compounds are usually generated from the degradation of lignin and are categorised into three classes of organic compounds, namely acids such as syringic acid and vanillic acid, aldehydes such as syringaldehyde, and ketones including vanillone (Chandel et al., 2011). Phenolic compounds are also natural components of some biomass materials such as sorghum and its bran (**Section 1.2.6.3**), grape pomace etc., and can thus be liberated into solution by the hydrolysate production processes. These phenolic compounds in sorghum bran have desirable bioactive properties and are highly sought after in foods, but can considerably inhibit ethanol fermentation (Pandey et al., 2014). The mode of action of these inhibitory compounds have been discussed in **Section 3.1.3**. Various studies have investigated the impact of inhibitory compounds on yeast fermentations (e.g. Converti et al., 1999), but much less is known about the effect of these inhibitory compounds on fungal fermentations with moulds such as *A. terreus*.

Detoxification of hydrolysates refers to the specific removal of any inhibitors present in a biomass hydrolysate prior to fermentation, by any of several physical, chemical or biological means (Palmqvist and Hähn-Hagerdal, 2000). Various such methods are now discussed below.

### **5.1.1 Physical Detoxification**

Physical detoxification involves techniques which do not utilise chemical reactions, with methods instead based on manipulation of the physical properties of the hydrolysates.

#### **5.1.1.1 Rotary Evaporation**

This involves the evaporation of volatile components, achieved by extraction of hydrolysates with organic solvents such as ether. The non-volatile fraction of a willow hemicellulose hydrolysate obtained by rotary evaporation, containing 2.4 g/l acetic acid and non-volatile phenolic compounds, was found to be inhibitory to ethanol fermentation decreasing productivity by up to 46 % (Palmqvist *et al.*, 1996).

### **5.1.1.2 Solvent Extraction**

This method utilises the selective solubility of the various constituents of a solution in an immiscible liquid solvent. It involves the use of a two-phase contact method to remove several extraneous compounds such as phenolics and other aromatic compounds, furancarboxylic acids and other chemicals in the hydrolysate (Luo *et al.*, 2002). Ethyl ethanoate extraction was reported to remove furfural, acetate and other inhibitors, resulting in an increase in fermentation yield by *Pichia stipitis* from 0 to 93% of the yields of a reference fermentation (Wilson *et al.*, 1989). A similar extraction of pine hydrolysate was found to increase glucose consumption rate by 12-fold with a suggestion that low-molecular weight phenolic compounds were the fraction with the greatest inhibitory activity in the ethyl ethanoate extract (Zhuang *et al.*, 2009).

The main advantage of solvent extraction is the ability to remove both volatile and non-volatile inhibitors from a hydrolysate while the main drawbacks are the need for an extra solvent removal step and that of biocompatibility with the fermenting organism (Blaschek *et al.*, 2010).

### **5.1.1.3 Ultrafiltration**

Membrane filtration is an approach for the separation of liquids into two fractions by passing them through a semi-permeable membrane. Ultrafiltration (UF) refers to a variety of membrane filtration techniques in which pressure or a concentration gradient leads to the separation through the membrane, with the high molecular weight solutes remaining in the retentate while low molecular weight solutes pass in water into the permeate/filtrate. The membrane used are film-forming polymer materials such as cellulose acetate and polypropylene and usually should have a pore size that is a tenth of the size of the particle that is to be separated to minimize blockage (Cooper, 2013).

Two main applications of UF in industrial biotechnology are the concentration and purification of enzymes and the clarification of fermentation broths where it can be used to separate low molecular weight substances such as glucose and ethanol from yeast biomass. The main advantages of UF in fermentation is the use of low temperatures, low energy requirements and can be used to concentrate or dilute a product. However, there are disadvantages such as the need to recourse to diafiltration (a sort of filter cake washing) and the low filtration rates (Cooper, 2013). Even though this method has not yet commonly been

used in hydrolysate detoxification as evidenced by the lack of scientific literature, it appears to show a lot of potential.

### **5.1.2 Biological Detoxification**

Biological detoxification involves treatment of biomass material with microorganisms such as fungi and bacteria or fungal enzymes, which are able to lower or remove inhibitory compounds.

#### **5.1.2.1 Fungi and Bacteria**

Fungi such as *Trichoderma reesei* have been employed in detoxifying hydrolysates and found to improve ethanol yield by up to 4-fold (Palmqvist *et al.*, 1997; Jönsson *et al.*, 1998). However, these fungi also utilise the sugars thus decreasing the available sugars for fermentation. Bacteria have also been employed. For example, a waste house wood acid hydrolysate was treated with the thermophilic bacterium *Ureibacillus thermosphaericus* to produce similar yields as hydrolysates treated by overliming in subsequent ethanol fermentation with *S. cerevisiae* (Okuda *et al.*, 2008). These authors report that this bacterium has the advantage over fungi in that they do not assimilate sugars to the same extent as fungi.

#### **5.1.2.2 Use of Enzymes**

Fungal enzymes such as peroxidases and laccases have been obtained from the ligninolytic fungus *Trametes versicolor*. Laccase treatment has been reported to lead to selective and virtually complete removal of phenolic monomers (originally present at 2.6 g/l in the crude hydrolysate) and phenolic acids

### **5.1.3 Chemical Detoxification**

#### **5.1.3.1 Overliming**

Chemical methods of detoxification include the overliming method which removes furfural and phenolic compounds. It involves raising the pH of acidic hydrolysates with  $\text{Ca(OH)}_2$  to pH 9-10 resulting in the precipitation of gypsum, followed by the restoration to pH 5.5 with

H<sub>2</sub>SO<sub>4</sub>. This method is reported to precipitate toxic compounds, and thus improve fermentability, more than standard NaOH neutralisation (Palmqvist, 1998 cited in Palmqvist and Hähn-Hagerdal, 2000). Furthermore, the combination of overliming and sodium sulphite addition has been shown to be very effective in the detoxification of willow hemicellulose hydrolysate prior to fermentation by recombinant *Escherichia coli* (Olsson *et al.*, 1995). The main drawback of this method is the accumulation of large quantities of gypsum, the product of the neutralisation.

#### **5.1.3.2 Adsorption (Activated Charcoal)**

Another method involves the adsorption of the inhibitory compounds using substances such as activated charcoal which can substantially reduce the levels of furans, weak acids and phenolics (Pandey *et al.*, 2014) because the compounds adsorb onto the surface of the charcoal. Activated charcoal was shown to remove over 95 % of phenolics from oak wood hydrolysate (Converti *et al.*, 1999) while 89 % of furfural was removed from sugarcane bagasse hydrolysate (Rodrigues *et al.*, 2001). This is a cheap and efficient way of removing inhibitors in hydrolysates. The effectiveness of activated charcoal treatment depends on various factors such as pH, contact time, temperature and the quantity of activated charcoal used (Prakasham *et al.*, 2009).

#### **5.1.3.3 Ion Exchange**

Ion exchange resins (IERS) are polymers which, when hydrated, dissociate to yield equivalent amounts of oppositely charged ions and are capable of exchanging these ions within the polymer with ions in a solution around them (Alchin, 2016). Strong acid and strong base resins refer to resins in the acid or base form that completely dissociate to give free hydrogen or hydroxide ions respectively in the presence of external counter-ions (Harland, 1994). Common exchangers are cation exchangers which include polystyrene resins with sulphonate groups, or anion exchangers such as resins with amine groups. They have been applied in chemical detoxification of hydrolysates where they are shown to remove various classes of inhibitors. Anion exchange resins were applied to the detoxification of sugarcane bagasse hydrolysate and it was observed that 84 % of the acetic acid was removed raising subsequent ethanol fermentation from 0.27 g/g sugar to 0.37 g/g (van Zyl *et al.*, 1991). They are particularly effective for the detoxification removal of lignin-derived inhibitors and acetic

acid in hydrolysates but are expensive and are thus not a cost-effective means of detoxification (Chandel et al., 2011).

All of the various detoxification methods described above are aimed at removing inhibitory compounds and improving fermentation yields. With regard to the present study, the red bran dilute acid hydrolysate has already been shown to contain small amounts of the known inhibitors HMF, furfural and ferulic acid (**Figure 3.9**). Given that little is known about the effect of these compounds on fermentations by *A. terreus*, it was considered worthwhile to investigate whether purification of the RBDAH might decrease the effect of inhibitory compounds in such a way to improve the fermentative production of itaconic acid from dilute acid hydrolysate. However, it is also important to note that the RBDAH promoted vegetative growth to a greater extent than a defined glucose medium (**Figure 4.27**). Therefore rather than a strict growth inhibition, it was also possible that factors in the RBDAH might have resulted in a nutrient balance favouring primary versus secondary metabolism, therefore inhibiting aspects of secondary metabolism. Thus, through refinement of the RBDAH it might be possible to overcome this effect. For example, high phosphorus content might have been responsible for the low itaconic acid production of sorghum flour as a feedstock (Petruccioli et al., 1999). Thus, intrinsic compounds such as mineral content and the polyphenol content of the sorghum bran and hydrolysates were also determined as these could also have an effect on a biocatalyst in fermentations.

#### 5.1.4 Aims

The aim of experimental work in this chapter was to investigate whether the use of physical and chemical methods of hydrolysate purification might improve the fermentation of the sorghum bran hydrolysate leading to higher levels of itaconic acid production by *A. terreus*.

Specific aims were to:

- Determine the most effective method for the purification of the RBDAH in terms of improvement in fermentation parameters such as yield and productivity
- Determine the types and content of pretreatment-induced and intrinsic inhibitors present in the hydrolysates before and after the treatments.

- Compare the various media produced in terms of itaconic acid fermentation output to determine the most suitable purification technique.

## **5.2 Materials and Methods**

The materials and methods specifically applied in this chapter are as detailed below, while general materials and methods have been described in **Chapter 2**.

### **5.2.1 Materials**

All materials and reagents used were of at least analytical grade.

#### **- Ion Exchange Resins**

The polar ion exchange resins used in this section are of various types and have different mesh sizes, ion exchange capacities (meq/ml), and degree (%) of divinylbenzene (DVB) resin cross-linkage, while non-polar resins are characterised based on dipole moment, pore size (Angstroms), mesh and surface area (sq. m/g). The polar resins include a strong acid cation exchanger, Dowex 50WX8 (50-100 mesh; 1.7 meq/mL; 8 % DVB), a weak acid cation exchanger, Dowex 50WX2 (50-100 mesh; 0.6 meq/mL; 2% DVB) and a weak base anion exchanger, Dowex 66 (50 mesh; 1.6 meq/mL). Non-polar XAD resins Amberlite XAD-2 (0.3 dipole moment; 20-60 mesh; 330 sq. m/g; 90 Å) and Amberlite XAD-16 (0.3 dipole moment; 20-60 mesh; 900 sq. m/g; 100 Å) were also used.

#### **- Hydrochloric acid (0.4 M)**

Eighty millilitres of 5 M HCl was added to 800 ml with reverse osmosis water, mixed and then made up to 1 L.

#### **- 20 % w/v Calcium hydroxide solution**

Twenty grams of  $\text{Ca(OH)}_2$  was made up to 100 ml in a volumetric flask and shaken thoroughly to ensure mixing.

#### **- 0.01N Hydrochloric acid solution**

In a volumetric flask containing 0.1 ml of 5 M hydrochloric acid (HCl) water was added and made up to a final volume of 100 ml.

**- 2 % (w/v) Anhydrous sodium carbonate solution**

Into a volumetric flask, 2.0 g of anhydrous sodium carbonate was transferred and made up to 100 ml with water. This was shaken thoroughly and used immediately.

**- 0.2 M Acetate buffer with 0.17 M sodium chloride**

Into a volumetric flask containing 80 ml of water, 1.143 ml of glacial acetic acid was transferred, and 0.994 g of sodium chloride added. The solution was mixed thoroughly and the pH was adjusted to 5.0 with NaOH solution and made up to 100 ml with water.

**- 1 % Vanillin in methanol**

Into 90 ml of methanol, 1.0 g of vanillin was added and made up to 100 ml with absolute methanol. The solution was stored in a dark bottle at 4°C.

**- 4% and 8 % Concentrated HCl in Methanol**

Exactly 8.0 mL of concentrated HCl was brought to 100 ml with methanol, capped and mixed carefully by inversion. This solution was diluted with equal volumes of methanol to prepare a 4 % solution.

**- 0.3 mg/ml Catechin solution**

Exactly 3.0 mg of catechin was dissolved in absolute methanol up to 10 ml. The solution was shaken vigorously to ensure mixing, and stored in a dark bottle at 4°C for up to three days.

**-Working vanillin reagent**

This was prepared fresh before use by mixing equal parts of the 1 % vanillin solution with the 8 % HCl solution.

## **5.2.2 Methods**

### 5.2.2.1 Hydrolysate Purification

In this section various approaches taken to purify the hydrolysate are described. The hydrolysates were then fermented and compared with a reference RBDAH fermentation.

#### - Activated Charcoal Pretreatment

Activated charcoal is a form of carbon which contains several tiny pores in its matrix, thus increasing the surface area available for adsorption, with 1 g possessing a surface area up to 3.0 km<sup>2</sup> (Dillon *et al.*, 1989). This characteristic was exploited in this section to investigate the potential of the activated carbon to adsorb any inhibitors present in the hydrolysate, in a bid to increase downstream itaconic acid yields.

Granular activated charcoal powder (Fisher Scientific) with a dimension of around 2.5 mm was prepared by washing with water twice and decanted. It was then equilibrated with 0.4 M hydrochloric acid until the powder stopped bubbling when it was then sieved, washed with water and dried at room temperature (Carvalho *et al.*, 2005). The activated charcoal was mixed with the RBDAH at a rate of 20 % w/v and shaken at 200 rpm for 3 h after which the slurry was filtered. The RBDAH was adjusted to pH 3.1 and sterile-filtered.

#### - Overliming

It has been suggested that during neutralisation, furfurals and phenolic compounds may, to some extent, be precipitated while overliming may also help to remove volatile inhibitors such as furans (Chandel *et al.*, 2011).

Here instead of adjusting with pellets or a concentrated NaOH solution (as in regular RBDAH preparation), the dilute acid hydrolysate was adjusted to pH 10.0 by the addition of a 20 % w/v solution of Ca(OH)<sub>2</sub>. The RBDAH was left for 1 h and then centrifuged at 4,000 rpm for 10 mins and decanted. The pH of this medium was then carefully re-adjusted to 3.1 with H<sub>2</sub>SO<sub>4</sub> then filter-sterilised (Larsson *et al.*, 1999) and stored at 4°C pending fermentation.

The pH of another batch of RBDAH was simply raised from ca. 1.0 to 3.1 using the Ca(OH)<sub>2</sub> solution and filter-sterilised and stored.



#### - Ethyl Ethanoate Extraction

Due to the specific ability of solvent extraction to remove phenolic compounds and other aromatics (**Section 5.1.1.2**), this method was investigated for the potential to purify (detoxify) the RBDAH.

Solvent extraction was performed according to an adaptation of the method previously described (Clark and Mackie, 1984). The RBDAH was mixed with equal volumes of ethyl ethanoate and shaken at 200 rpm for 1 h then the aqueous fraction collected. The process was repeated thrice and the aqueous fractions pooled together and heated at 65 °C to remove the last traces of ethyl ethanoate, as it was not possible access a rotary evaporator it at the time. The extracted RBDAH was then sterile filtered and stored at 4°C pending fermentation.

In addition, the ethyl ethanoate fractions from the extraction of RBDAH were also pooled together, dried using an air stream. The residue was dissolved in hot water (about 70 °C) to the same concentration as the bran i.e. 10 % SL by making 20 mg of the residue up to 200 ml.

#### - Ultrafiltration

This method utilised a membrane filter which was attached to a rotor pump that pumped hydrolysate through the filter from one end, such that while the permeate was collected at the other end, the retentate flowed back into the source vessel.

#### - Use of Ion Exchange Resins

Both cation and anion exchangers were employed in an attempt to exchange any inhibitory ions with innocuous ones. It was anticipated that these would replace the various cations and anions with the innocuous  $H^+$  and  $OH^-$  respectively which will then combine to form water thus purifying the hydrolysate.

Various classes of exchange resins were employed. Strong cation exchanger Dowex 50WX8, weak cation exchanger Dowex 50WX2, and the weak anion exchanger Dowex 66 were used. In addition, non-ionic hydrophobic resins Amberlite XAD-2 and Amberlite XAD-16 were employed in the purification attempts, all in batch experiments. The resins were washed with methanol and swelled for 1 hr. They were then rinsed in water and filtered four times before soaking till use. Each resin slurry was filtered and then mixed with the hydrolysate and the

pH of the resin-hydrolysate mix adjusted to 10. The mixture was incubated at room temperature and shaken at 160 rpm for 90 min then the pH adjusted to 3.1 with H<sub>2</sub>SO<sub>4</sub>. The hydrolysates were filtered, sterilised and then employed in fermentations.

#### - Alkaline Tannin Removal

Tannin removal has been reported to directly improve ethanol yields (**Section 1.2.6.3**) and it was hypothesised that this might be the case with itaconic acid fermentation too. Attempts were made to reduce the tannin content of the hydrolysates by the alkaline soaking method using dilute sodium hydroxide as follows: duplicate 4 % w/v slurries of red bran were prepared by making 20 g of red bran up to 500 ml with 0.05M NaOH solution. They were incubated at 30 °C for 24 hr, the contents were then vacuum-filtered (Chavan *et al.*, 1979). The residue was then rinsed several times with sterile distilled water until the pH was around 7.0, then filtered and air-dried. The dry detannified bran was then used to prepare a dilute acid hydrolysate as described in **Section 3.3.4**.

#### - Phosphate Removal

The effect of the phosphate content of the hydrolysate on the fermentation process was investigated. The phosphate was removed from the hydrolysates using a compound known as PhosGuard<sup>TM</sup> (Seachem, USA), which are aluminium oxide-based spherical beads which work by the irreversible binding of phosphates (and silicates) at the interface of chemical and water. According to the manufacturers it does not release any ions into the liquid because it is not an exchange resin (Seachem, 2016). The PhosGuard<sup>TM</sup> beads were dropped into a beaker of water to wet them, then removed with a spatula and transferred into bottle containing red bran dilute acid hydrolysate at pH 3.1 at a rate of 0.005 % w/v. The bottle was then capped and shaken at 200 rpm at ambient temperature for 24 h to allow the beads adequate contact with the liquid. The beads were then removed by filtration and the phosphorus content of both RBDAH and RBDAH treated by phosphate removal (RBDAH-PO<sub>4</sub><sup>-</sup>) determined by elemental analyses using ICPMS as described in **Section 5.2.2.2** and compared.

All the hydrolysates obtained from the various detoxification processes were fermented for itaconic acid production as described in **Section 2.2.4** for 7 days with samples collected daily for analysis.

#### **5.2.2.2 Determination of Intrinsic Inhibitors**

Specific components of the red bran dilute acid hydrolysate which could potentially inhibit the itaconic acid production were determined as presented below. These tests were performed on the hydrolysates obtained.

##### **- Total Phenolic Compound Content**

All sorghum varieties contain phenolic compounds which are compounds with one or more hydroxyl groups directly attached to an aromatic ring and include the flavonoids, tannins and phenolic acids. The total polyphenols content in the RBDAH, RBDAH treated by the various detoxification methods and the solution of the ethyl ethanoate extract were determined by the Folin-Ciocalteu (F-C) test as follows. First, an aliquot of the sample was diluted ten-fold with water then to 0.1 ml of this, 2.0 ml of freshly prepared 2 % (w/v) anhydrous sodium carbonate was added. The mixture was vortexed vigorously then left to stand for 5 min. Afterwards, while vortexing again, 0.1 ml of a fresh 50 % solution of F-C reagent was carefully added to it then left to stand for between 40 min and the absorbance read (Vermerris and Nicholson, 2007). The absorption was read from 500 - 900 nm to determine the maxima for *p*-coumaric and ferulic acids and this was used to measure the samples. The blank was a solution of 0.1 ml water, sodium carbonate solution and F-C reagent.

Standard curves were plotted and the phenolic compound contents of the samples read as ferulic and *p*-coumaric equivalents. The results are presented as the phenolic acids removed and these were calculated thus:

$$\text{Phenolic acids removed} = \left\{ \frac{\text{P.a. equivalent of hydrolysate}}{\text{P.a equivalent of RBDAH}} \right\} \times 100$$

#### - Condensed Tannin Content

In addition to phenolic compounds, sorghum cultivars with a pigmented testa also contain tannins. Condensed tannins are flavonoid-based while hydrolysable tannins are gallic-acid based, but both groups still display considerable chemical variability (Vermerris and Nicholson, 2007). The tannin forms found in sorghums are condensed tannins, whereas hydrolysable tannins or tannic acids are not usually present in sorghum (Dykes and Rooney, 2006). The condensed tannins in the hydrolysates were determined by the vanillin/HCl assay with commercial catechin standards (catechin is a flavan-3-ol which can form the building block of condensed tannin). This is the best method for the estimation of condensed tannins as the absolute tannin content of sorghum is impossible to determine because a significant proportion cannot be extracted (Hahn et al., 1984; Butler, 1990 cited in Rooney, 2012) and no suitable commercial standards for tannins are available (Rooney, 2012).

Into two sets of culture tubes, 0 to 1.0 ml of the catechin standard are dispensed and each sample adjusted to 1 ml with absolute methanol and tubes incubated in a 30°C water bath. Exactly 5.0 ml of the working vanillin reagent was added at 1 min intervals to one set of standards and 5.0 ml of the 4 % HCl added similarly to the second set of standards (both solutions had previously been brought to 30°C in the bath too). The samples were left for exactly 20 min in the bath before being read at 500 nm absorbance. Care was taken to ensure the 1 min intervals were maintained when reading samples to ensure exactly equal times are allowed for colour formation in the samples. The blank used was the vanillin reagent with no catechin and a standard curve plotted.

For sample analysis, 1.0 ml aliquots of each hydrolysate or extract were dispensed in duplicates into culture tubes which were then similarly treated as the standards described above. The absorbance values obtained for the blanks set were then deducted from values of the samples set and compared to the standard curve to obtain the condensed tannin content as “catechin equivalents” (Price *et al.*, 1978). The results were presented as condensed tannins removed as a percentage of the original, similar to the approach used for total phenolic compound content as directly above.

#### - Elemental Analyses

The composition of the elements in the red sorghum bran hydrolysate was determined in a multi-element analysis to specifically estimate the phosphorus content of the RBDH and the

PhosGuard™ treated hydrolysates, in order to determine the effect of treatment on itaconic acid fermentation. This was performed by Inductively Coupled Plasma Mass Spectrometry (ICPMS) using a Thermo-Fisher iCAP-Q equipped with CCTED (collision cell technology with energy discrimination (Thermo-Fisher Scientific, USA).

Multi-element analysis was undertaken at the school of Veterinary Medicine laboratories using a 'Flatopole collision cell' (typically charged with helium gas) upstream of the analytical quadrupole to reduce polyatomic interferences. Internal standards were normally introduced to the sample stream via a T-piece and typically included Sc ( $50 \mu\text{g l}^{-1}$ ), Ge ( $20 \mu\text{g l}^{-1}$ ) Rh ( $10 \mu\text{g l}^{-1}$ ) and Ir ( $5 \mu\text{g l}^{-1}$ ) in the preferred matrix of 2% HNO<sub>3</sub>; a similar concentration of HCl was also acceptable. External calibration standards are usually all in the range 0 – 100  $\mu\text{g l}^{-1}$  (ppb). Samples were introduced via a covered autosampler (Cetac ASX-520) through a concentric glass venturi nebuliser (Thermo-Fisher Scientific) or a PEEK Burgener Miramist nebuliser. Sample processing was undertaken using 'Qtegra software' (Thermo-Fisher Scientific) Results were reported as gravimetric concentrations ( $\mu\text{g/l}$  or  $\text{mg/l}$ ) in Microsoft Excel spreadsheet format (UoN, 2015).

#### **5.2.2.3 Itaconic Acid Fermentation**

Following attempted purification (detoxification) of the various hydrolysates, the resulting refined hydrolysates were used in itaconic acid fermentations with *A. terreus* isolate 49-22 and the outputs presented as percentages of the theoretical maximum yields based on sugar consumed (see **Section 4.2.2**). The original RBDAH was included as a control, with triplicate fermentations performed for each purified hydrolysate sample.

## 5.3 Results

### 5.3.1 Effect of Hydrolysate Detoxification on Hydrolysate Parameters

Various physical and chemical detoxification strategies were performed on RBDAAH which were aimed at producing hydrolysates with improved characteristics for fermentation.

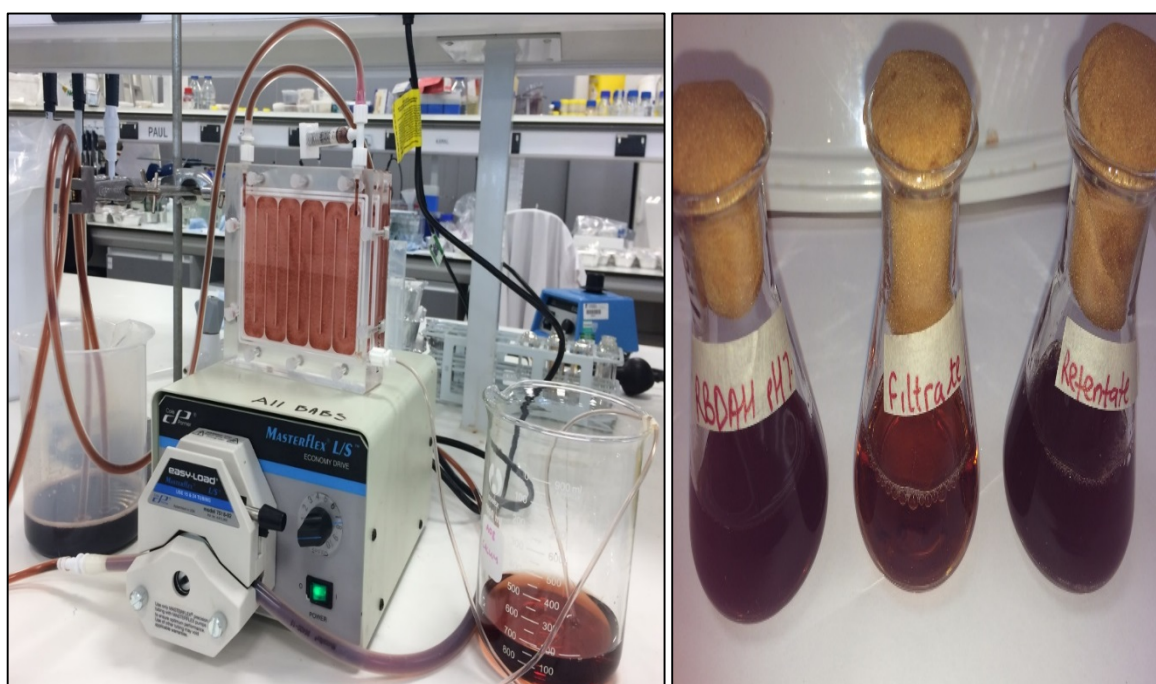
#### 5.3.1.1 Appearance of Hydrolysates

The colour of the hydrolysates varied considerably with the various treatments as shown in **Figure 5.1** below. The Dowex ion exchangers completely transformed the hydrolysates from red to a cream to yellow colour. Activated charcoal also changed the colour of the RBDAAH to a lighter shade of red.



**Figure 5.1:** The appearances of the various red bran dilute acid hydrolysates after treatment by various detoxification methods. From left to right: regular (NaOH treated) RBDAAH, lime neutralised RBDAAH; overlimed RBDAAH; activated carbon treated RBDAAH; ethyl ethanoate extracted RBDAAH; dowex 66 treated RBDAAH; dowex 50WX2 treated RBDAAH; dowex 50WX8 treated RBDAAH; amberlite XAD16 treated RBDAAH; and amberlite 2 treated RBDAAH.

The ultrafiltration was performed at pH 7.0 in order to protect the membrane which resulted in a very dark red hydrolysate (as the colour of the RBDAH deepens with a rise in pH). The process produced a retentate which appeared on visual examination to be considerably darker than the original RBDAH, while the filtrate/permeate was clearest (**Figure 5.2**). The detannified hydrolysate was a considerably lighter shade of red than the RBDAH (result not shown), as the red colour of the bran leached into the 4 % solution of NaOH during the process (**Section 5.2.2.1**) resulting in a lighter bran obtained after drying.



**Figure 5.2:** *Left:* The ultrafiltration set-up utilised in the RBDAH purification process. The left beaker contained the original RBDAH and the final retentate, while the permeate was collected in the left beaker. *Right:* The effect of ultrafiltration on the appearance of the fractions of RBDAH

### 5.3.1.2 Glucose Content of Hydrolysates

The glucose contents of the hydrolysates obtained from the various purification processes were estimated by HPLC (**Section 2.2.6**) and found to be different from the original RBDAH. The glucose content of each new medium is presented as a function of that present in the RBDAH (**Table 5.1**), by subtracting the RBDAH from it and expressing the difference as a percentage of the RBDAH's glucose content.

**Table 5.1:** Effect of the various detoxification treatments on glucose content of red bran dilute acid hydrolysate.

HYDROLYSATE	GLUCOSE CONTENT (g/l)	GLUCOSE CHANGE (%) <sup>*‡</sup>
RBDAH	51.62	0.00 <sup>a</sup>
Lime neutralised RBDAH	51.02	-1.16 <sup>b</sup>
Overlimed RBDAH	38.64	-17.22 <sup>c</sup>
Activated Charcoal treated RBDAH	51.21	-0.79 <sup>a,b</sup>
Ethyl ethanoate extracted RBDAH	62.48	21.04 <sup>d</sup>
Detannified RBDAH	42.98	-16.74 <sup>c</sup>
Ultrafiltration retentate	46.58	-9.32 <sup>f</sup>
Ultrafiltration permeate	52.60	2.34 <sup>g</sup>
DOWEX 66 treated RBDAH	47.85	-7.30 <sup>h</sup>
DOWEX 50WX2 treated RBDAH	53.7	4.03 <sup>i</sup>
DOWEX 50WX8 treated RBDAH	55.94	8.37 <sup>j</sup>
AMBERLITE XAD2 treated RBDAH	56.72	9.88 <sup>k</sup>
AMBERLITE XAD16 treated RBDAH	53.24	3.14 <sup>g</sup>

\*: Hydrolysates with different superscripts have statistically significant differences in their glucose values.

‡ Negative values indicate a decrease in glucose content after the treatment.

Of the 12 hydrolysates investigated, six showed a drop in glucose content compared to the RBDAH, with the greatest change observed being in the overlimed RBDAH with a loss of over 17 % followed by the RBDAH produced by the detannification process with a decrease in glucose content of 16.7 % compared to the regular RBDAH. By contrast, increases of 3 % and 21 % were observed with the AMBERLITE XAD16 and the ethyl ethanoate treatments, respectively. Ultrafiltration caused a significant decline in the glucose content of the retentate while the permeate showed an increase relative to RBDAH.

### 5.3.2 Intrinsic Inhibitors

Some compounds are part of the chemistry or composition of a biomass material and may be leached or released into the hydrolysate during pretreatment where they then retard or inhibit fermentation processes. Common examples of these are soluble compounds derived from the



biomass material itself, degradation products of the carbohydrates and lignin, and heavy metal ions that could come from soil attached to the biomass or even be a part of the biomass itself. For sorghum bran, phenolic compounds are a good example of these intrinsic inhibitors, while phosphorus in high amounts in sorghum could also inhibit itaconic acid fermentation. Experimental work found the following levels of such substances.

#### **5.3.2.1 Total Phenolics Content**

The amount of phenolic compounds present in the RBDAH and the various hydrolysates was determined by the Folin-Ciocalteu method. This method is a colourimetric method originally used to detect tyrosine and tryptophan in protein hydrolysates but is now widely used to determine phenolics in various sources (Vermerris et al., 2007). The total phenolic compounds of the RBDAH are presented in mg/ml and the amount removed from by the various treatments as related to the amount in the original RBDAH are presented in **Table 5.2**. It was observed that the various hydrolysates had different phenolic acid (*p*-coumaric and ferulic acid) equivalents spanning a wide range. The baseline was the RBDAH with *p*-coumaric and ferulic acid equivalents of 38.1 and 32 mg/ml, respectively. The treatments were all found to change the content of phenolic equivalents in each hydrolysate to a similar extent (%) regardless of the standard referred to. The most successful treatment in bringing down the phenolic acid content was the detannification process which achieved a nearly 74 % removal rate. Other successful treatments include the use of DOWEX 50WX2 and Amberlite XAD16 which removed about 64 % and 65 %, respectively, and activated charcoal treatment with an approximate 42 % decrease. The ethyl ethanoate extracted RBDAH showed just 8.8 % decrease in phenolic compounds; the residue containing the extracts from the RBDAH was dissolved in water and also tested for polyphenolic compounds, and it was found to contain approximately 90 % less than (or 10 % of) the RBDAH phenolics content in *p*-coumaric acid equivalents. The phenolic content of the ultrafiltration fractions were compared with the RBDAH at pH 7 to eliminate any effects of the neutralisation on the readings. It was observed that the retentate had a 24.6 % increase in phenolics with the permeate showing a near-similar decrease with 27.4 % removal of phenolics.

**Table 5.2:** Total phenolic compounds in the various hydrolysates as determined by the Folin-Ciocalteu test and phenolic removal (combined *p*-coumaric and ferulic acid values).

HYDROLYSATE SAMPLE <sup>y</sup>	<i>P</i> -COUMARIC ACID EQUIVALENT (mg/ml)	FERULIC ACID EQUIVALENT (mg/ml)	PHENOLIC REMOVAL (%)*‡
RBDAH	38.10	32.00	0
Lime neutralised DAH	19.52	16.40	48.76
Overlimed DAH	19.05	16.00	50.01
Activated charcoal treated DAH	22.20	18.65	41.73
Ethyl ethanoate extracted DAH	34.76	29.20	8.76
Detannified DAH	10.00	8.40	73.75
Ethyl ethanoate extract solution	3.83	3.22	89.95
DOWEX 66 treated DAH	34.11	28.65	10.47
DOWEX 50WX2 treated DAH	13.81	11.60	63.75
DOWEX 50WX8 treated DAH	29.00	24.36	23.88
AMBERLITE XAD16 treated DAH	13.33	11.20	65.00
AMBERLITE 2 treated DAH	33.17	27.86	12.94
<b>ULTRAFILTRATION</b>			
RBDAH (pH 7)	48.15	40.45	0
Ultrafiltration retentate (pH 7)	60.00	50.40	-24.61
Ultrafiltration permeate (pH 7)	34.95	29.36	27.41

‡ Negative values indicate an increase after the treatment.

<sup>y</sup>: All hydrolysates were adjusted to pH 3.1 with sulphuric acid and sodium hydroxide before the analysis

### 5.3.2.2 Condensed Tannin Content

The tannins found in sorghum are condensed tannins, with no hydrolysable tannins present. These compounds were estimated in the various hydrolysates using the vanillin-hydrochloric acid method and results are presented below (**Table 5.3**). The hydrolysates were detannified to various extents, with the Dowex 66 treatment removing over 90 % of the condensed

tannins in the hydrolysate while Dowex 50WX2 removed over 78 %. Less successful treatments include lime neutralisation and ethyl ethanoate extraction, which each only removed 12.5 % of the condensed tannins in the RBDAH. The tannin removal rates were found to be significantly different from the RBDAH and from one another ( $F = 3079.17$ ;  $DF = 14$ ;  $P = 0.0000$ ) for every pair of treatments, except for the Amberlite XAD 16 treatment of RBDAH and detannification detoxification processes which removed the same amounts of tannin (75 %); the activated charcoal and overliming treatments (37.5 %); and the lime neutralisation and ethyl ethanoate extraction treatments (12.5 %). All the resin treatments were very successful, removing over half of the condensed tannins with the least being Dowex 50WX8 with a removal of 31.3 %. The ultrafiltration was performed with RBDAH previously adjusted to pH 7 with NaOH, and the condensed tannin content of the fractions were determined and compared with the RBDAH at pH 7 to eliminate any effects of the neutralisation on the readings. The results showed that the process was successful as the permeate had a 28.9 % removal of CTs while the retentate showed a much higher CT content of 34.1 % (**Table 5.3**).

**Table 5.3:** Condensed tannin (CT) content of the purified hydrolysates as determined by the Vanillin-HCl method.

HYDROLYSATE SAMPLE <sup>γ</sup>	CATECHIN	CT REMOVAL
	EQUIVALENTS (mg/l)	
		(%)* <sup>‡</sup>
RBDAH	72.04	0
Lime neutralised DAH	63.03	12.50
Overlimed DAH	45.02	37.50
Activated charcoal treated DAH	45.02	37.50
Ethyl ethanoate extracted DAH	63.03	12.50
Detannified DAH	18.01	75.00
Ethyl ethanoate extract solution	86.90	-20.63
DOWEX 66 treated DAH	6.75	90.63
DOWEX 50WX2 treated DAH	15.76	78.13
DOWEX 50WX8 treated DAH	49.53	31.25
AMBERLITE XAD16 treated DAH	18.01	75.00
AMBERLITE 2 treated DAH	35.5	50.72
<b>ULTRAFILTRATION</b>		
RBDAH (pH 7)	33.77	0
Ultrafiltration retentate (pH 7)	45.29	-34.11
Ultrafiltration permeate (pH 7)	24.01	28.88

<sup>‡</sup> Negative values indicate an increase after the treatment.

<sup>γ</sup>: All hydrolysates were adjusted to pH 3.1 with sulphuric acid and sodium hydroxide before the analysis

### 5.3.2.3 Elemental Analyses

The determination of the phosphorus content of the hydrolysate was considered paramount because it has been reported that high phosphorus content might be responsible for low itaconic acid production when using sorghum flour as a feedstock (Petrucchioli et al., 1999). Phosphorus, alongside other elements in the red bran dilute acid hydrolysate was analysed by ICPMS (Section 5.2.2.2). Subsequently, an attempt was made to decrease the phosphate content of the red bran dilute acid hydrolysate using PhosGuard<sup>TM</sup> beads (Section 5.2.2.1) and the effect of the treatment evaluated for elemental phosphorus. The results are presented in Table 5.4 below. Please note that it was not possible to use replicate measurements (to allow statistical validation of data) due to the high costs of the ICPMS analysis.

Fourteen elements were determined in the hydrolysates and it was observed that there were high amounts of sodium, magnesium and sulphur in the RBDAH. Other main elements detected included phosphorus, potassium and calcium. Seven other elements were detected in trace quantities notably iron and zinc. A relatively high sodium content was detected in the RBDAH (21.6 g/l) which was removed almost completely by the phosphate removal process. By contrast, the aluminium content of the RBDAH-PO<sub>4</sub><sup>-</sup> increased from 4.6 mg/l to 180.9 mg/l following purification. Very surprisingly, the phosphate content in the PhosGuard<sup>TM</sup> bead treated fraction actually showed a 21% increase compared to the original RBDAH (increase from 0.38 to 0.46 g/l; Table 5.4).

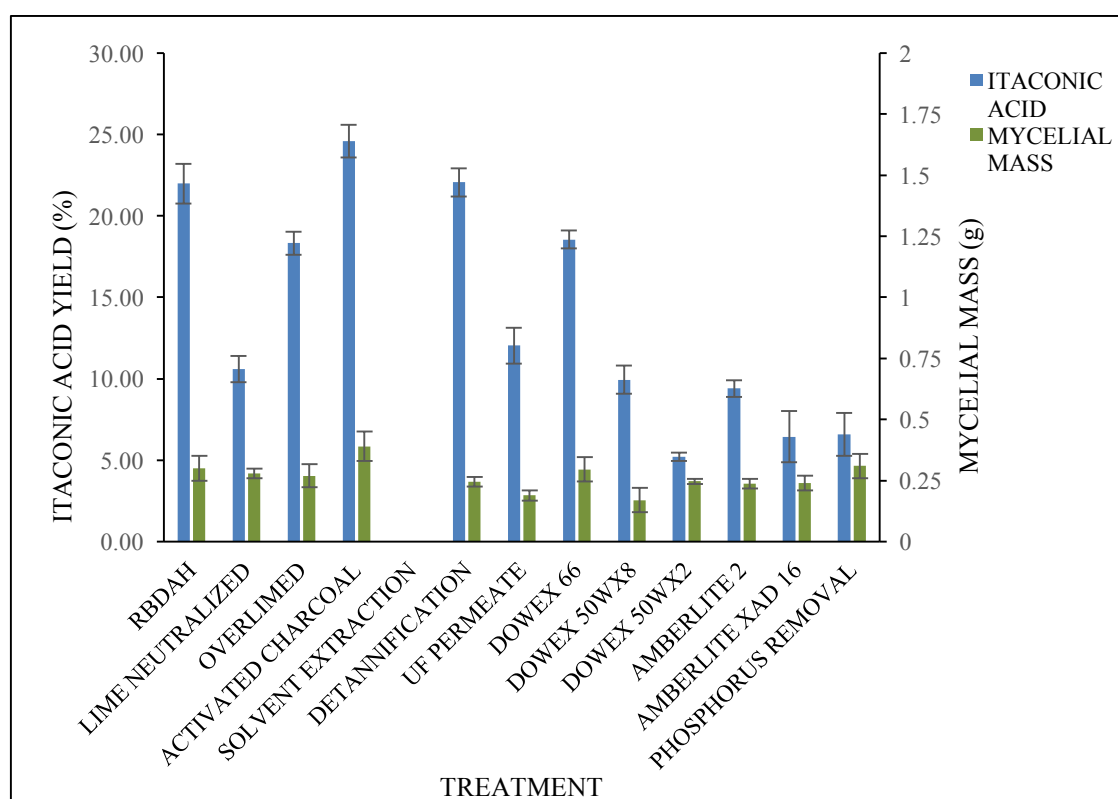
**Table 5.4:** Multi-element analysis performed by Inductively Coupled Plasma Mass Spectrometry (ICPMS) using a Thermo-Fisher iCAP-Q system equipped with collision cell technology with energy discrimination (CCTED).

Element	Na	Mg	P	S	K	Ca	Ti	Al	Mn	Fe	Co	Ni	Cu	Zn
Unit	g/l	g/l	g/l	g/l	g/l	g/l	g/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
<b>RBDAH</b>	21.6214	1.1546	0.3806	29.6491	0.7762	0.2921	0.0000	4.5762	5.3604	20.0577	0.0373	0.2252	4.6234	12.6
<b>RBDAH – PO<sub>4</sub><sup>-*</sup></b>	0.2549	0.5276	0.4609	28.9155	0.5465	0.2694	0.0001	180.8666	8.9798	115.3118	0.0491	0.1044	2.8675	54.2

\* This hydrolysate has been treated with PhosGuard™ beads in a batch process to attempt to remove the phosphates in the red bran dilute acid hydrolysate (RBDAH)

### 5.3.3 Itaconic Acid Fermentation

The various hydrolysates generated were employed in itaconic acid fermentation with *A.terreus* 49-22 as described in Section 4.2.2. The various fermentations were sampled daily and HPLC analysis showed that they proceeded at different rates, thus concluding on different days (results not shown). The highest yields (g/l) obtained over the seven day sampling period for each of the various hydrolysates was used to calculate the yield as percentage of the theoretical maximum (See Section 4.2.2.3) which are presented in Figure 5.4 below. It was observed that there were indeed differences in the yields of the various hydrolysates and the values are presented in Figure 5.4 below.



**Figure 5.4:** Effect of various detoxification strategies on the yields of itaconic acid on red sorghum bran dilute acid hydrolysate (RBDAH) by *Aspergillus terreus* 49-22 at 37 °C and 200 rpm for 7 days. Yield values are the peak values obtained over the 7-day period and are presented here as a percentage of the theoretical maximum possible based on glucose consumed.

One way ANOVA showed that there was a significant difference ( $F= 1578.00$ ;  $df = 11$ ;  $P=0.0000$ ) between the itaconic acid yields of the various RBDAHs. The activated charcoal treatment resulted in a yield of 24.6 % of theoretical maximum itaconic acid possible, surpassing that of the RBDAH at 22.0 %. Overliming was also successful in improving the fermentation, although the 0.1 % increase was not statistically significant. Detannification was the only other treatment that resulted in comparable itaconic acid yields with the RBDAH, producing 22.1 % of the theoretical maximum possible. The other treatments were mostly detrimental to the fermentation process. The resin treatments generally resulted in lower yields ranging from 5.2 % to 18.5 % of maximum itaconic acid possible, while the Dowex 50 WX2 treatment produced the least itaconic acid. It was observed that the ethyl ethanoate extraction did not allow the growth of *A. terreus* and correspondingly did not produce any itaconic acid.

Multiple range test using Fisher's least significant difference (LSD) procedure showed that there were significant differences between 62 possible combinations of treatment pairs (**Appendix 4**). These included the RBDAH versus activated charcoal fraction, an important result showing that IA yields could be marginally increased. The exceptions were the RBDAH-overliming pair, Amberlite XAD16 and phosphorus removal, and the detannification-RBDAH sets of treatments where there were no differences. There was no significant difference between mycelial mass observed indicating that (with the exception of the ethyl ethanoate solvent extraction) the treatments were similar in terms of ability to support *A. terreus* 49-22 growth ( $P = 0.0001$ ).

## 5.4 Discussion

The red bran dilute acid hydrolysate was subjected to various physical and chemical processes aimed at improving its fermentation characteristics. The resulting purified hydrolysates were evaluated for their glucose and possible inhibitor contents, and also their fermentation yields.



## 5.4.1 Effect of Detoxification on Hydrolysates and Hydrolysate Parameters

### 5.4.1.1 Appearance of Hydrolysates

The treatments changed the appearance of the RBDAH to various extents based on visual examination, with the most considerable being the Dowex ion exchangers. Dowex 66 resulted in a light yellow appearance which was similar to the RBEH (**Figure 3.10**). The bright red colour of the RBDAH (at the acidic pH it is produced in) is derived from the bran itself which contains all the pericarp and other components of the grain, with lutoforol being the main pigment. Pigmentation in the pericarp is due to the presence of phenolic flavonoid pigments called anthocyanins and flavan-4-ols and the colour intensity has been reported to be dependent on pH. Anthocyanins are very unstable in acidic pH and are readily converted into the corresponding anthocyanidins under low pH conditions (Rooney, 2012). The DOWEX 66 ion exchanger is widely used for the de-ashing of high fructose corn syrup and other starch-based sweeteners (DOW, 2016b). Phenolic compounds are weak acids but with a  $pK_a$  value of 10, they are weaker than carboxylic acids (Vermerris and Nicholson, 2007). Loss of a  $H^+$  from the hydroxyl group results in the formation of a phenoxide ion. It can thus be deduced that, being a weak base anion exchanger the DOWEX 66 ion exchanger was able to remove the anions in the hydrolysate including this weak acid phenoxide ion of the phenolic compounds. Thus the hydrolysate would be expected to have a greatly reduced phenolic compounds content, which thus explains the clearer appearance.

The strong acid cation exchangers DOWEX 50WX2 and DOWEX 50WX8 differ in the level of cross-linking with the former containing a lower degree crosslinking with divinylbenzene (2 %) than the latter (8 %). The more tightly cross-linked a resin is, the lower its permeability; DOWEX 50WX8 is thus less permeable than DOWEX 50WX2 but it has a higher exchange capacity (DOW, 2016a). The effect of the differing cation exchange capacities and permeabilities were employed in the RBDAH purification (detoxification) and it was observed from visual examination, that RBDAH treated with DOWEX 50WX2 was clearer than that treated with DOWEX 50WX8. This implied that the higher cross linking could inhibit the ability of DOWEX 50WX8 to bind to positively charged colour compounds even though it had a higher cation exchange capacity. The change was however not as profound as with DOWEX 66.

Amberlite XAD-2 and -16 are hydrophobic polyaromatic non-polar resins and are recommended for adsorption of organic substances up to MW 20,000 and MW40,000 respectively from aqueous systems (Sigma-Aldrich, 1998). They were investigated for ability to adsorb and thus remove the compounds like vanillin, vanillic acid and furans, as a natural hydrophobic compound (zeolite) has been shown to successfully remove them from hydrolysates (Ranjan *et al.*, 2009). Successful removal of these compounds was expected to result in a lightening of the RBDAH appearance. However, even though slightly clearer, this was not particularly evident as can be seen in **Figure 5.1**.

Other non-resin treatments included activated carbon treatment which also works by adsorption. This method using non-polar activated charcoal is commonly used to remove non-polar molecules in decolorisation processes because such charcoal traps various organic chemicals as well as inorganic chemicals such as chlorine via Van de Waal's forces. It is thus likely that the charcoal has adsorbed some of the red pigments of the RBDAH, accounting for the visible change in the appearance in **Figure 5.1**. Lime neutralisation, overliming and ethyl ethanoate extraction did not cause significant changes in the hydrolysate's appearance. During the detannification process, the red colour of the bran leached considerably into the dilute NaOH used, and resulted in a lighter bran after drying. This subsequently produced a light reddish-orange hydrolysate which was similar to the Amberlite-treated RBDAHs.

If the red and other phenolic compounds in the hydrolysate inhibit *A. terreus* fermentation linked to IA production, the lighter detoxified hydrolysates were expected to show improved fermentation characteristics.

#### **5.4.1.2 Glucose Content of Hydrolysates**

Various detoxification processes have been shown to result in considerable sugar losses, which may negate any benefits accruable from the improved yields due to the detoxification. For instance with the overliming process, severe sugar losses have been reported at pH 11 (Mohagheghi *et al.*, 2006) while long contact times can cause over 70 % glucose loss (Millati *et al.*, 2002). Similarly, activated charcoal at high loadings can cause over 31 % glucose loss (da Silva *et al.*, 1998). Consequently, the sugar content of the red bran dilute acid hydrolysate was investigated before and after the purification treatments.

It was found that while seven treatment processes did not cause a significant decline in amounts of glucose, six processes resulted in glucose loss in the final hydrolysate (**Table 5.1**). The most detrimental process for sugar retention was overliming which caused a 17.2 % drop in glucose content. It has previously been reported that hydroxide-catalysed degradation and conversion of sugar into unfermentable products can occur, with a 14 % loss of glucose reported by other authors (Amartei and Jeffries, 1996; Mohagheghi *et al.*, 2006). Similarly, losses of 34% and 21% have been reported for xylose and arabinose respectively (Mohagheghi *et al.*, 2006) due to the overliming treatment. This extensive sugar degradation by overliming could be due to the stabilisation of reactive enolate intermediates by calcium ions (Jonsson *et al.*, 2013). Thus, overliming is a really harsh method of detoxification in this regard.

The hydrolysate produced from the alkaline detannification process was also lower in glucose content than the reference RBDAH, although it was necessary to use a different batch of RBDAH for this analysis and it is acknowledged that there might have been slight batch to batch differences. However the 16.7 % decrease in glucose content was high enough to infer with some certainty that the detannification contributed to the lower glucose content. Glucose loss from RBDAH was not statistically significant with both lime neutralisation and activated charcoal treatments. This decrease was lower than the 6.4 % glucan loss reported for bagasse after lime pretreatment (Chang *et al.*, 1998), and this might be due to the different applications of the lime in conditioning the feedstocks.

As a that the hydrolysate would be heated at 65°C, a temperature close to the boiling point of ethyl ethanoate (77.1°C) but sufficiently mild to minimise evaporation and inhibitor formation. The ethyl ethanoate extraction process resulted in a 21 % increase in glucose content of the RBDAH, and this might be ascribed to the heating process which although low, still caused evaporative loss of 25 % of the hydrolysate volume (results not shown), thus concentrating it. Thus, the apparent major increase in glucose content of the ethyl ethanoate extraction was perhaps an artefact. This effect was also observed in vacuum evaporation where detoxified wood hydrolysate was reduced to 1/3 of the original volume (Parajó *et al.*, 1997).

Differences in glucose content were evident in the different fractions collected from ultrafiltration whereby the permeate had a higher glucose content than the retentate, although the permeate level was only marginally higher than the original hydrolysate. This indicates

that glucose was capable of passing through the membrane into the permeate. Purification using cation exchangers and non-polar resins also caused a slight increase in glucose content. Although the resins were hydrated before use, it is possible that further swelling might have occurred following exposure to the hydrolysates resulting in a removal of water, and consequent increase in glucose concentration in solution. However, the anion exchanger Dowex 66 caused a statistically significant 7.3 %, loss of glucose, similar to the 8 % loss recorded using AG 1-X8 anion exchanger to detoxify spruce hydrolysates at pH 5 (Larsson *et al.*, 1999). The activated charcoal treatment resulted in 0.8 % decrease in glucose content which was considerably better than values of up to 26.5 % glucose loss reported by Villarreal *et al.* (2005). This loss could be due to hydrophobic interactions of glucose and the divinylbenzene-based matrix (Larsson *et al.*, 1999).

Overall, methods that result in minimal loss of or increase in glucose content were considered likely to possess an advantage in terms of use of fermentations to increase IA yield over methods that resulted in glucose loss.

## **5.4.2 Intrinsic Inhibitors**

### **5.4.2.1 Total Phenolics Content**

All sorghums contain phenolic compounds including flavonoids and phenolic acids, but not all contain the third type of compounds, tannins. The Folin-Ciocalteu (F-C) test was conducted to determine the total phenolic contents of the various hydrolysates. These compounds are usually produced during hydrolysate preparation as lignin degradation products are leached from the cereal/biomass itself. *P-coumaric* and ferulic acids were chosen as standards against the more commonly used gallic acid because ferulic acid are the most abundant phenolic acids in cereal grains such as barley (Bartolomé *et al.*, 2003) and in sorghum.

High polyphenols content in sorghum has been associated not only with poor nutritional quality in animals, but with poor malting and brewing characteristics due to their ability to bind to and thus inhibit the action of relevant enzymes. It has also been shown that polyphenols in birdproof (brown) sorghums can be inhibitory to amylolytic enzymes [Daiber, (1975) and Kock *et al.* (1985) cited in de Jong *et al.* (1987)]. Tannins are the major

polyphenols in sorghum (Dunford, 2012) and they have been reported to range from 0 – 10.2 % catechin equivalents in sorghum grains (Shahidi and Naczki, 2003). It was thought possible that they could bind to the enzymes in the itaconic acid fermentation process to retard yields. A simple alkaline removal process exists to remove tannins and this was performed as described (**Section 5.2.2.1**).

It was observed that the alkaline detannification process resulted in an RBDAH with 74 % less *p*-coumaric and ferulic equivalents than the original. This result is statistically significant compared to the hydrolysate prepared with pre-detannification, and is similar to results obtained by Chavan *et al* (1979) who reported 75 - 85 % tannin removal using NaOH and KOH in a similar method. A similar cold soaking treatment in sodium bicarbonate was also shown to remove over 53 % of tannins from black gram (*Vigna mungo*) (Shah, 2001). Lime neutralisation removed about 49 % of phenolics which surpassed the 19 % removal reported by Larsson *et al.* (1999). Another alkaline treatment tried was overliming of the RBDAH and it was found that it removed half of the total phenolics content of the RBDAH. This is higher than reports for overliming of sugarcane bagasse at 35 % and 36 % decrease in phenolics respectively (Martinez *et al.*, 2001; Chandel *et al.*, 2007). Overliming is described as one of the most efficient methods of hydrolysate detoxification (Jönsson *et al.*, 2013) where it removes volatile inhibitory compounds such as furans (Chandel *et al.*, 2007), even though the exact mechanism of action is still debated. It has been suggested that detoxification was by the precipitation of toxic substances (Van Zyl *et al.*, 1988) but analysis of the precipitates and hydrolysates have indicated a chemical conversion of toxic substances as opposed to precipitation (Persson *et al.*, 2002).

The ethyl ethanoate extraction removed 8.8 % of the phenolics from the RBDAH and upon resuspending the residue obtained from the extraction (in water to the same concentration as the hydrolysate) it was found to contain about 90% less phenolics content than the RBDAH. This confirmed that the ethyl ethanoate did extract the phenolic compounds from the RBDAH. However, a higher decrease was expected from the extraction process and this poor outcome may be due to the subsequent drying process which increased the concentration of residual phenolics (which are non-volatile in nature) in the RBDAH. This suggestion is supported by the fact that glucose was also found to be higher in the extracted RBDAH thus indicating a concentration process. Low success in the detoxification by solvent extraction of steam-exploded hydrolysate of poplar wood followed by subsequent low enzymatic saccharification rates and ethanol production are also reported (Cantarella *et al.*, 2004).

Activated charcoal treatment of RBDAH resulted in the removal of 41.7 % of the total phenolics of the RBDAH and this was close to the range of the 51.2 – 87.5 % reported for various hydrolysates in literature (Villarreal *et al.*, 2006; Chandel *et al.*, 2007). The resin treatments were the most effective at total phenolics removal as the Dowex 50WX2 and Amberlite XAD16 treated RBDAH were very effective in removing 63.8 and 65 % of the total phenolics. Similar high results have been reported (Villarreal *et al.*, 2006) and the better performance of ion exchange resins over activated charcoal and other methods has been previously reported (Chandel *et al.*, 2007). The anion exchanger Dowex 66 and less crosslinked Amberlite XAD2 removed about 10 % and 13 % of phenolics, respectively.

The application of ultrafiltration was successful in phenolics removal resulting in an increase of 24.6 % in phenolics in the retentate while the permeate showed a commensurate decrease of 23.3 %. As was expected the RBDAH for this process, which was held at neutral pH to protect the membrane, was a darker red colour than all the other hydrolysates; but it was observed that this resulted in significantly higher phenolic acid reading between the same batch of RBDAH at pH 3.1 and at 7.0 with the FC test despite the care that was taken to select the various blanks used in the readings. Thus, there appears to be an inherent difficulty in using this spectrophotometric process to analyse deeply-coloured hydrolysates. It could also account for the overall high values observed in this test for all the hydrolysates.

#### **5.4.2.2 Condensed Tannins Content**

Some sorghum varieties in addition to the two common phenolic compounds (flavonoids and phenolic acids) contain tannins. Condensed tannins (CT) are of particular importance among the phenolic compounds because they are the only types of tannins found in sorghum, and because of their ability to bind to and precipitate proteins such as enzymes (Dunford, 2012; de Jong *et al.*, 1987). Their presence in the RBDAH was investigated and the effect of various detoxification methods on its content were evaluated.

All treatment methods resulted were found to reduce the amounts of condensed tannins in amounts ranging from 9.4 % - 90.6 %. The most effective method was the use of the Dowex 66 anion exchanger which gave over 90 % removal while all the other resin treatments also worked well. This is in complete agreement with a previous report that anion exchange was most effective at removing phenols and also furans from spruce hydrolysate (Nilvebrant *et al.*, 2001). Overliming and activated charcoal showed similar activity in CT removal, both

removing 37.5 % of the CTs from hydrolysates. By contrast, the ethyl ethanoate extraction and lime neutralisation removed only 12.5 % each of the tannins. This relatively poor outcome may be due to incomplete removal of the solvent from the RBDAH during the drying process which proved to be inhibitory to the *A. terreus*. It could also be due to the concentration of the RBDAH due to evaporation (as evidenced by the higher glucose content) which means higher amounts per ml of the condensed tannins. The RBDAH at pH 7 showed a much lower amount of CTs with 33.8 catechin equivalents than at pH 3.1 with 72 mg/l catechin equivalents and the reason for this is not immediately obvious. The neutral RBDAH was used to determine the removal rate of the ultrafiltration method and the results show that it resulted in a decrease of 28.9 % in the permeate and a corresponding increase of 34.1 % in the retentate.

This work is believed to be the first report on the investigation of the effect of purification/detoxification processes on condensed tannins in biomass hydrolysates.

#### **5.4.2.3 Elemental Analyses**

A multi-element analysis of the RBDAH and the PhosGuard<sup>TM</sup>-treated RBDAH were performed using Inductively Coupled Plasma Mass Spectroscopy, a technique capable of detecting several elements, including metals, down to very low concentrations of parts per billion (UoN, 2015). The findings (**Table 5.4**) show that Na content was considerably high in the RBDAH at 21.6 g/l, which was attributed to the neutralisation process. The sorghum bran hydrolysates are highly buffered and thus required large quantities of sodium hydroxide to raise the pH to desired levels. The phosphate removal process however almost completely removed the Na, leaving just 0.2 g/l. Similarly, other elements including Mg, K and Ca were reduced in quantity by the process. However, several other elements showed an increase, notably Al which rose from 4.6 mg/l to 180.9 mg/l, most likely due to the leaching of aluminium oxide from the alumina beads into the hydrolysate [the manufacturers cautioned that although aluminium does not leach into solution at neutral pH, it can do so at low pH levels (Seachem, 2016)]. Phosphorus levels also increased significantly from 0.38 g/l to 0.46 g/l after the treatment which was unexpected as the reverse effect was expected. The failure of this treatment to remove phosphate might be due to the fact that the present work used the PhosGuard<sup>TM</sup> beads outside of their recommended applications of phosphate removal in marine and freshwater aquaria. However, the phosphorus levels from the ICPMS process

were determined to be over 16.5 times higher than levels present in the defined medium. This may indicate that while the RBDAH contains adequate phosphorus for vibrant *Aspergillus terreus* growth, the composition might be too rich for optimal itaconic acid production.

### 5.4.3 Itaconic Acid Fermentation

The various hydrolysates generated were all used in fermentations with *A. terreus* and sampled daily for analysis of itaconic acid (IA) production. The peak outputs were then used to calculate the yields as a function of glucose consumed up to that point. The hydrolysates contained different levels of glucose and other compounds/ions, thus the fermentation proceeded at varying rates, peaking and terminating at different times (results not shown).

The benchmark RBDAH fermentation produced itaconic acid at an efficiency of 22 % of the theoretical maximum possible. A key result was therefore that almost all the treatments attempted resulted in lower conversion efficiencies, which was disappointing as it had been anticipated that increases in IA yield might have been possible. These findings contrasted with those, for example, of Nilvebrant *et al.* (2001), who found that the anion exchanger AG 1-X8 resulted in a spruce hydrolysate with better productivity than a reference fermentation. The only exceptions were with treatment of the RBDAH using activated charcoal and the hydrolysate prepared following alkaline detannification of bran, which resulted in better yields of 24.6 and 22.1 % respectively with the former being statistically significant. Activated charcoal treatment actually resulted in a higher concentration of itaconic acid produced (9.04 g/l), but the glucose consumed was also high (51.62 g) (results not shown), thus decreasing the yields based on glucose consumption. By possible way of explanation, the activated charcoal treated RBDAH also supported more vigorous *A. terreus* growth (0.39 g) than the hydrolysate produced from the detannified red sorghum bran (0.25 g) and regular RBDAH (0.3 g), which may account for the high glucose consumption with less than commensurate itaconic acid production. However, there was no overall significant difference between mycelial mass according to types of purification of the hydrolysate.

One final observation to highlight was the possible effect of phosphate levels on IA yield. It has previously been reported that high phosphorus content might be responsible for low itaconic acid production when using sorghum flour as a feedstock (Petruccioli *et al.*, 1999). It was found that the inadvertent 21 % increase in phosphate levels when using the PhosGuard<sup>TM</sup> beads was correlated with a 70 % fall in IA yield, suggesting that phosphate



content might indeed significantly suppress itaconic acid production (whilst having an insignificant effect on mycelia formation). However, this hypothesis requires further investigation as changes in the levels of several other nutrients occurred during the PhosGuard™ treatment which may have also been contributory factors.

#### **5.4.4 Summary and Conclusions**

Sorghum grain has desirable properties such as drought resistance, high starch content and low price which gives it a high potential for fermentative production of value-added products. This chapter investigated various methods for conditioning the dilute acid hydrolysate obtained from red sorghum bran in order to obtain a more favourable hydrolysate for the production of itaconic acid. Unfortunately time did not allow for parallel studies with the white bran hydrolysates nor to see the effects of purification on ethanol yield from yeasts.

- The various methods used resulted in hydrolysates with different physical appearances and chemical compositions. The processes resulted in varying degrees of glucose loss or gain relative to the control RBDAH.
- All treatments resulted in a decrease in total phenolic compounds compared to the original RBDAH, with between approximately 9-90 % removal possible. Most treatments also were effective in removing condensed tannins from the RBDAH with between approximately 12-90 % removal possible.
- Fermentation with these media showed that changes in chemical composition of the hydrolysate following the treatments resulted in changes in IA yields by *A. terreus*.
- The use of activated charcoal and detannification treatments resulted in higher overall IA yields than the control defined glucose medium. This suggested the successful removal of potentially inhibitory components from the sorghum bran hydrolysate. However, the increases were not found to be significant, and all other treatments resulted in decreased IA production.

## **Chapter 6: Strain Improvement and Optimisation of Itaconic Acid Fermentation Process**

### **6.1 Introduction**

Since the discovery of penicillin (**Section 1.2.1**) tremendous efforts have gone into the production of, and screening for, secondary metabolites. However, the manufacture of commercially important metabolites from wild-type organisms is usually economically unfavourable (Han and Parekh, 2005). This problem has in many cases been overcome by the application of traditional strain-improvement methods followed by fermentation process optimisation. For every improved strain obtained, the fermentation process will be optimised to determine the optimum fermentation conditions for maximum product formation with the new strain.

A fermentation process is aimed primarily at producing a product that will sell at a profit and this implies that even processes that can assure an increase in output may not be economically viable, and so will not be implemented. Free-living microbes producing novel compounds of commercial interest in their natural surroundings will generally not make the product in economically viable quantities as in nature because metabolism is strictly controlled to minimise wasteful expenditure of energy or wasteful accumulation of metabolic intermediates (Parekh, 2009).

For every useful microbial product, it is therefore usually necessary to develop a strain which meets among others, a number of key requirements:

- The ability to effectively utilise inexpensive and complex substrates
- The presence of short fermentation times with lower demands on utilities e.g. air, cooling water and electricity
- The presence of altered product ratios in order to over-produce desired compound while minimising by-product formation
- Tolerant of high product concentrations and prevention of catabolite repression
- High viability and inhibitor tolerance combined with genetic stability (Gad, 2007).

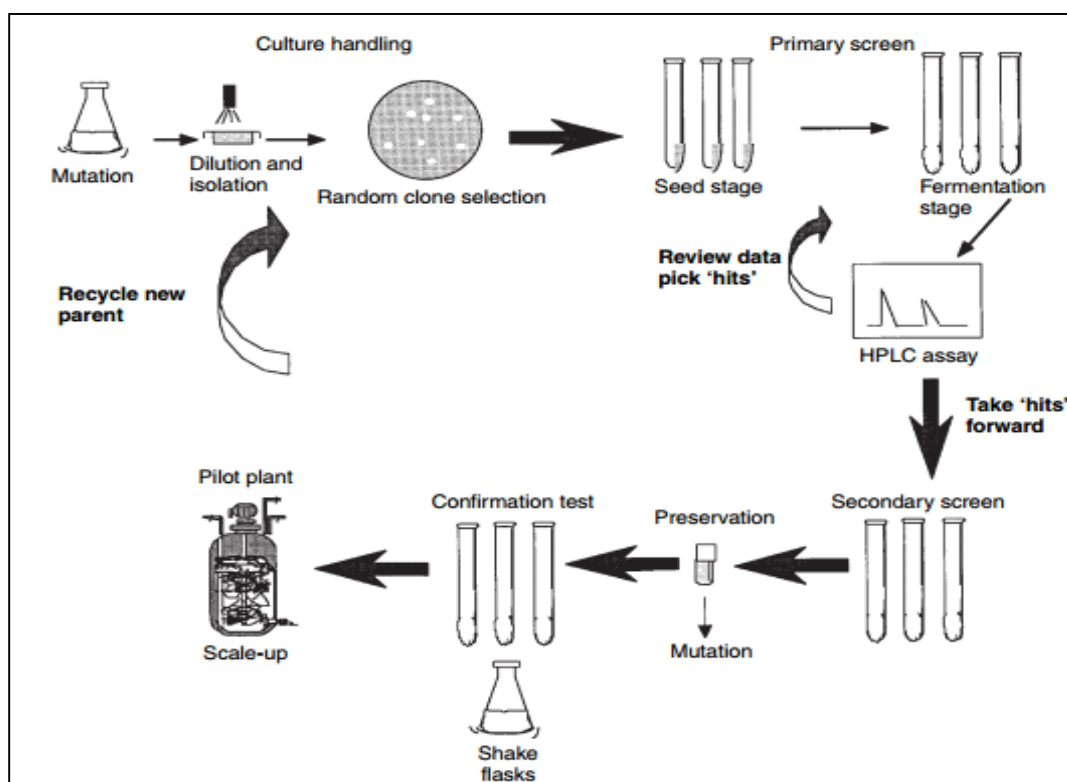
These all keep the cost of raw materials and downstream processing costs to a minimum (Hunter, 2007; Parekh, 2009). Strain improvement programs always target one or more of these desired phenotypes. Strain improvement is thus defined as the modification of a strain for overproduction of bioactive compounds, in a bid to making the fermentation process more cost-effective. It involves designing, breeding and continuously improving the performance of microbial strains for biotechnological applications (Hunter, 2007).

The tight genetic and metabolic regulation and synthesis of bioactive compounds is controlled by the genetic make-up of the organism and in strain improvement this is manipulated to bypass and reprogram the regulatory controls. There are two main categories of strain improvement which both bring about a change in the DNA sequence, namely classical strain improvement strategies and recombinant techniques.

#### **6.1.1 Classical Strain Improvement By Mutation**

Conventionally, strain improvement is achieved through random mutation and selection processes in what is termed the classical approach. This method has been in use for over half a century with the best known example being the titre increases recorded with penicillin fermentation (Han and Parekh, 2005). The classical strain improvement approach involves the repetition of three steps:

- (1) **Mutagenesis:** This is the first key step of this approach and involves the generation of a mutant population by exposing the microorganism to a mutagen (**Figure 6.1**). Mutagens include X-rays, ultraviolet (UV) light, and chemicals such as N-methyl-N'-nitro-N-nitrosoguanidine. Mutagens operate through various mechanisms of action including, DNA breakage, base transitions, transversion, additions and deletions (Han and Parekh, 2005). Various mutagens may be evaluated until the most effective is discovered at generating desired diversity in the genome of the mutant population. These mutants acquire an altered genetic code with a “reprogrammed” biosynthetic capability, which could present in the phenotype in addition to the genotype of the mutant progeny.



**Figure 6.1:** A typical mutation and random selection process in microbial strain improvement (Parekh, 2009).

It is important to determine optimum mutation levels that maximise frequency of survivors with the desired yield-related mutation; and avoid ‘undermutagenesis’ which produces survivors with no genetic alterations, or ‘overmutagenesis’ where survivors accumulate too many mutations (Han and Parekh, 2005, Parekh, 2009).

(2) Screening: This involves the assessment of survivors of mutagenesis for improvement in phenotype. First the mutants are grown in solid/liquid media, single colonies are randomly selected and employed in small-scale fermentations. The main challenge of this random method is the high throughput required, i.e. the question of how many colonies are required to increase the chances of picking the desired improved mutant progeny. This depends on availability of time, manpower and equipment capacity. An improvement on the random screening method is the rationalised selection process which requires a basic understanding of the fermentation pathway and product formation which can then be exploited to select specific mutants. For example, environmental conditions of pH and temperature can be manipulated or chemicals introduced in culture media to select mutants with desired traits. This method

is a prescreening technique and can lead to effective mutant selection. The selected progeny are then subjected to fermentation to select strains with desired phenotype e.g. increased productivity (Han and Parekh, 2005, Parekh, 2009).

(3) Metabolite quantification and identification of improved strains: Mutants which show an increase in metabolite yield are considered potential “hits” and these are employed in several replications and the yield compared to check for statistically significant improvements over parental strains. The improvements are more likely to be small increments in output than drastic increases (Han and Parekh, 2005, Parekh, 2009). Upon confirmation, these improved strains from the primary run can then be used as parents for a repeat of the mutagenesis cycle to further improve the strain in so-called ‘enhancer screens’ (Dyer *et al.*, 2017).

The advantages of the classical strain improvement approach include

- Simplicity
- There is no need for prior knowledge of the biochemical pathway
- Requires minimal equipment and knowledge of the biosynthetic pathway or genetics of the microbe.

The main disadvantage is the large number of strains needed to be screened and low chances of successful hits, because it works by non-specific non-targeted gene mutations. However, this is being overcome by extensive automation resulting in high throughput screening, and this method still remains the primary improvement strategy for any new strain improvement program (Hunter, 2007).

### **6.1.2 Genetic Recombination By Classical Processes**

Most microorganisms are able to exchange genetic information by recombination involving a crossing-over event(s) thus resulting in a new sequence of nucleotides along the DNA which can result in stable, inheritable genetic traits. This process of gene alteration and strain modification is called genetic recombination and includes various classical techniques such as protoplast fusion, transformation, conjugation and sexual reproduction (Hunter, 2007;

Parekh, 2009; Ashton and Dyer, 2016). These methods have advantages over classical mutational methods in that they allow a more targeted approach (Parekh, 2009). Recombination can be guided to produce mutant strains with desirable properties e.g. one strain may produce high yields of the product, while another will have high inhibitor tolerance, and yet another might be able to utilise novel sugars. Genetic recombination offers the possibility to “construct” strains with combinations of these traits.

#### **6.1.2.1 Protoplast Fusion**

Protoplasts are cells whose cell walls have been removed with lytic enzymes. Fusing two closely related protoplasts combines their entire genetic material thus allowing the mixture of DNA independent of sex factors or genetic incompatibility to form a transient hybrid. This method has been widely used in brewing, and for the modification of several industrial strains of bacteria and fungi. The main advantage of this process is high frequency of recombinant production with up to 20 % reported in *Streptomyces coelicolor*. Also, up to four strains can be fused to yield recombinants with traits from all four parents. This technique has also been employed in penicillin strain improvement programs (Parekh, 2009; Stanbury *et al.*, 2013).

#### **6.1.2.2 Transformation**

This process involves the uptake of purified, exogenously supplied DNA which may exist independently or fuse with the DNA of the recipient cells or protoplasts (Parekh, 2009). This results in changes in the amount and sequence of the recipient’s DNA leading to increased chances of desirable traits. Transformation can involve genomic DNA or cloned sequences. This method allows the transfer of genetic material between unrelated strains and organisms. Transformation methods for strain improvement for production of primary or secondary metabolites have been demonstrated in *Streptomyces*, *Bacillus*, *Saccharomyces*, and *Aspergillus* species.

#### **6.1.2.3 Conjugation**

This involves the unidirectional transfer of genetic material between strains. It is mediated by plasmid sex factors and requires cell contact and DNA replication. This technology has been employed in improvement of *Lactococcus* strains in the dairy industry (Parekh, 2009).

#### 6.1.2.4 Sexual Reproduction

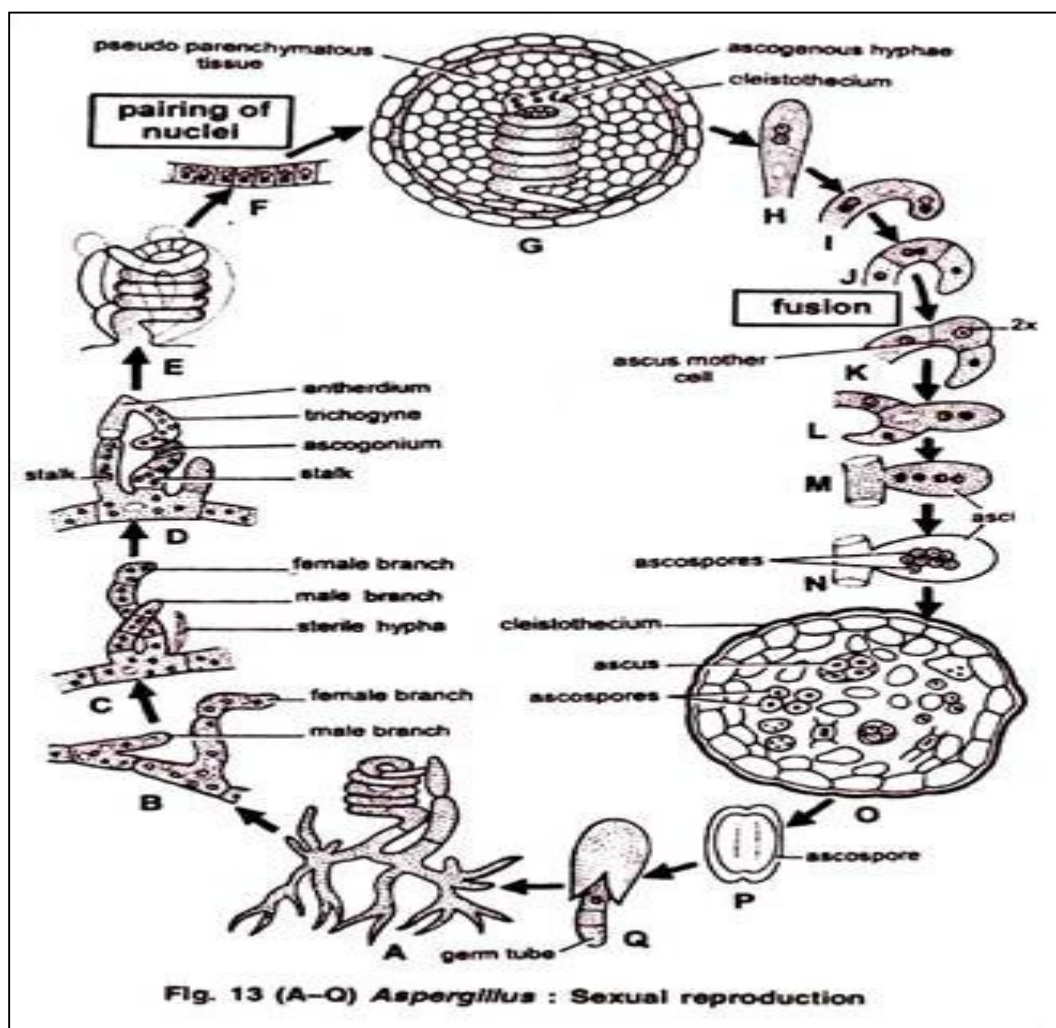
Sexual reproduction is often overlooked but this method is also an important method of gene transformation, and genetic recombination by sexual reproduction has also been employed in strain improvement (Ashton and Dyer, 2016). This section focuses on the process as it relates to filamentous ascomycete species (pezizomycetes), which include industrially important fungi such as *Aspergillus terreus*.

#### Overview

Fungal sexual reproduction involves a cycle of plasmogamy (fusion by gametes or gametangia) followed by karyogamy and meiosis. Sexual reproduction exists in all fungal phyla, except in the Glomeromycota (Alexopoulos and Mims, 1979). Fungal microscopic sexual structures were first described in 1820 in a culture of *Syzygites megalocarpus* while in 1904 the mating types of *Rhizopus nigricans* were elucidated and the terms “homothallism” and “heterothallism” were coined to describe self-compatible and self-incompatible (or obligate outcrossing) individuals (Ehrenberg, 1820, Blakeslee, 1904 cited in Houbraken and Dyer, 2014). Up to 20% of fungi lack known sexual cycles and have traditionally been lumped together as the Deuteromycota or Fungi Imperfecti. However, recent advances in genomic studies have resulted in the discovery of mating-type genes and sexual cycles for several previously considered asexual species, leading to the reclassification of these strains (Dyer and O’Gorman, 2011; Dyer and O’gorman, 2012).

In the case of heterothallic ascomycete species it is necessary for isolates of compatible ‘mating type’ to be present for sexual reproduction to occur. Such isolates are morphologically indistinguishable, but differ in the production of mating pheromones and receptors as a result of the presence of different ‘mating-type’ (*MAT*) genes in the genome in a region known as the idiomorph (Houbraken and Dyer, 2014). The compatible mating types can be thought of as analogous to ‘male’ and ‘female’. The different mating types are known conventionally in pezizomycete species as *MAT1-1* and *MAT1-2* (other species e.g. *Neurospora* have alternate terminology eg *matA* and *mata*) (**Figure 6.2**), (Houbraken and Dyer, 2014). In *Aspergillus* species, these mating types have to come into close proximity for fusion to occur. The first stage involves the female ascogonium and the male antheridium, both haploid nuclei, which come together in a process called plasmogamy. Next the nuclei

divide and move to the hyphal tip forming dikaryotic ascogenous hyphae which form a hook called crozier tip following which karyogamy or fusion of the nuclei occur, and an ascus is formed from the tip. This is immediately followed by meiosis and mitosis to result in eight nuclei encased in the ascus. In filamentous ascomycetes, fruiting bodies such as cleistothecia in *Aspergillus* species and perithecia in *Neurospora crassa* surround the asci (Figure 6.2). Extensive discussions on sexual reproduction in fungi can be found in literature (Leslie, 1993; Chen and McDonald, 1996; Dyer, 2007; O’Gorman *et al.*, 2009; Arabatzis and Velegraki, 2012; Böhm *et al.*, 2013; Swilaiman *et al.*, 2013).



**Figure 6.2:** The sexual cycle in *Aspergillus* species (Khandelwal, 2016).



## **Environmental Requirements for Sexual Reproduction**

Sexual reproduction in fungi normally requires a specific set of environmental factors (light, temperature, nutrients etc.) which vary according to the individual species in question. For example, light has been shown to influence sexual reproduction in *A. nidulans* with incubation in the dark resulting in earlier cleistothecia formation and denser fruit bodies. Cleistothecia formation and density is also influenced by air exchange and wrapping Petri dishes with tape has been shown to double the amount of cleistothecia (Zonneveld, 1977). In addition, various nutrients influence the degree of sexual fertility and a solid surface is required for sexual development (Braus et al., 2002; Dyer and O’Gorman, 2012).

The conditions required vary from species to species within the genus *Aspergillus* and the slim chance of this specific microenvironment occurring in nature for sustained periods may explain why some species of *Aspergillus* and many other fungi still do not have a known sexual cycle in the wild, nor have one being successfully created in lab conditions. The sexual stage of *Aspergillus terreus* was not known and it was considered asexual, until Eagle (2009) showed that it harboured *MAT 1-1* and *MAT 1-2* genes and the requisite pheromone precursors for sex indicating a potential for sex, although attempts to cross various isolates of different mating type yielded no sexual structures (Dyer and Eagle, unpublished results). However, Arabatzis and Velegraki (2012) later reported the induction of a teleomorphic stage, characterised by cleistothecia formed inside hyphal masses, upon incubation of isolates of opposite mating types on a mixed cereal agar for three weeks.

## **Sexual Reproduction as a Method of Strain Improvement**

Sexual reproduction results in the fusion of the specialised sex cells of the fungus to give a diploid form which undergoes meiotic recombination along the whole genome (Nevalainen, 2001). Gene expression is altered by setting up crosses between parental strains with desired traits and then selecting progeny with the desired phenotype (Houbraken and Dyer, 2014; Ashton and Dyer, 2016). As a result, sexual crosses can be used to create fresh genetic diversity, with the offspring being screened for isolates showing either improved or reduced production of a particular metabolite and this phenomenon is referred to as “transgressive segregation” (Houbraken and Dyer, 2014; Ashton and Dyer, 2016). For example, the sexual cycle has been used to produce *Penicillium chrysogenum* progeny that secreted high titres of penicillin, without the contaminant chrysogenin (Böhm *et al.*, 2013). It also has a potential

for use in the development of new variants of blue-cheese with the recent discovery of sexual reproduction in *Penicillium roqueforti* (Swilaiman, 2013; Ropars *et al.*, 2014). The main drawback of this method is that it takes a long time to complete due to varying cross incubation times, but this is offset by the advantage of genome-wide recombination and the attendant genetic diversity and possibility of obtaining progeny with improvement in the trait of interest (Houbraken and Dyer, 2014; Ashton and Dyer, 2016). The success of exchange of genetic material can be verified by investigating genetic markers present in the parents and progeny. A number of different DNA fingerprinting methods such as randomly amplified polymorphic DNA (RAPD), arbitrarily primed (AP), and DNA amplification fingerprinting (DAF) polymerase chain reaction (PCR) have been used to characterise parents and progeny of crosses (Dyer *et al.*, 1996; Kumari and Thakur, 2014).

### **6.1.3 Genetic Recombination by Cloning and Genetic Engineering**

Techniques for manipulating DNA have progressed remarkably in the past few decades. It has become possible to break a DNA molecule at specific sites to isolate a DNA segment(s) and insert it at a specific point in another DNA molecule to create a recombinant DNA in a process called ‘genetic engineering’ or ‘genetic manipulation’ (GM) (Parekh, 2009). This in vitro recombinant DNA technology has various advantages over classical methods of strain improvement, principally being that it again allows a much more targeted approach. For example, the application of recombinant technology and the use of synthetic DNA now make it possible to induce specific mutations in specific genes. This process of inducing a mutation at a specific site in the genome is called site-directed mutagenesis. This process is invaluable in improving strains by improving/enhancing the catalytic activity of enzymes e.g. penicillin G amidase (Parekh, 2009). However, a major draw-back of these GM methodology is the high level of knowledge of the biochemical pathway and expertise required to undertake this approach. Reviews on the application of GM technology are available (Chater, 1990; Elander, 2003; Gavrilescu and Chisti, 2005). It is also noted that GM approaches have recently been applied to attempt to increase the production of itaconic acid by *A. terreus* and other microorganisms such as *S. cerevisiae* and *A. niger* (Li *et al.*, 2012; Blazeck *et al.*, 2014; Huang *et al.*, 2014). However further background is beyond the scope of the present study.

#### 6.1.4 Optimisation of Fermentation

All the methods discussed so far are strain improvement methods which target the microbial catalyst alone. While a high-yielding fungal strain is vital, the fermentation process itself also involves several other factors which can influence yields and this process can be optimised to improve the economics of the process. Optimisation of conditions can be described as the most important aspect of a fermentation process as it ensures that the most efficient use of resources are made, while maximising the yield of the desired product. The aim of fermentation process optimisation is to minimise the costs of manufacture by optimising the raw materials used e.g. substrates/nutrients, acids/alkalis, air etc. and energy use, while maximising efficiency of product formation, concentration and quality in the broth. Fermentation optimisation can thus be achieved with the judicious manipulation of both the physical and chemical parameters of the process (Smith, 2012).

The traditional method of optimisation is the one-variable-at-a-time (OVT) method, in which some variables are held constant while one variable is varied and its optimum found. The process is repeated varying each variable while holding the others at the optima that have been found until all the optima are found. This method is easy to perform but time-consuming and inefficient. In addition, the OVT does not take interactive effects between variables into consideration [Geiger, 1997 cited in de Cássia Lacerda Brambilla Rodrigues *et al.* (2012)]. Fermentation optimisations are now instead usually performed using statistical approaches which are quicker and more efficient (de Cássia Lacerda Brambilla Rodrigues *et al.*, 2012). Statistical design of experiments (DoE) is a mechanism of data collection appropriate for the study of a fermentation/biotechnological processes which has been made possible by the proliferation of commercial software packages to screen a large number of factors, such as media components, using a Plackett-Burman screening design of factorial design. This is then followed up by process optimisation. There are various statistical approaches for process optimisation but the best method is response surface methodology (RSM) which is very widely applied in industry especially where several variables potentially influence the output of the product process [Geiger, 1997 cited in de Cássia Lacerda Brambilla Rodrigues *et al.* (2012)]. RSM is a method of statistical optimisation based upon factorial designs, a collection of statistical and mathematical techniques that can be used to develop, improve and optimise a process. It determines optimum conditions by combining precise experimental designs with first and second order equations (Lacerda Brambilla Rodrigues *et al.*, 2012). The RSM is a very powerful method as it gives information about the

interaction between variables and requires relatively minimal amounts of experiments. Also, it can generate an empirical polynomial model (usually second order) which can be used to predict the levels of variables that can be used to obtain a maximum response output (Parekh *et al.*, 2000).

The use of RSM in optimisations have been shown to increase product yields in various biotechnological applications. For example, lipase production by *Pseudomonas aeruginosa* and *Aspergillus carneus* was shown to increase 1.8 and 5.6-fold after RSM optimisation, respectively (Kaushik *et al.*, 2006; Ruchi *et al.*, 2008).

After determining optimised conditions, the fermentation process can then hopefully be performed under economically viable conditions. In their discussion of antibiotic fermentation processes, Kuenzi and Auden, 1983 (cited in Smith, 2012) presented a range of constraints to various essential parameters of the fermentation process and the consequences (**Table 6.1**). All these factors need to be considered during the selection of process conditions. Various parameters which affect itaconic acid fermentation have been discussed in **Section 4.1.2.5**.

## 6.2 Aims

The overall aim of work in this chapter was to improve the concentration and yield of itaconic acid when using *A. terreus* with sorghum bran as a feedstock for fermentations. The specific aims were:

- To attempt to use the classical strain improvement technique of ultraviolet mutagenesis to screen for strains of *A. terreus* with improved itaconic acid production.
- To determine whether it is possible to employ sexual reproduction to induce genome-wide recombination in order to select for recombinant strains that might have altered itaconic acid fermentation properties such as improved yields or better conversion rates. The effect of various environmental parameters such as air exchange, temperature and medium type were also investigated to determine if sexual fertility could be enhanced.
- To optimise the itaconic acid fermentation process using experimental and statistical design methods.

**Table 6.1:** Constraints determining the design of industrial fermentation processes [Kuenzi and Auden, 1983 (cited in Smith, 2012)].

Process essential	Constraint	Possible Consequences
Microorganism	Nutritional requirements	High costs, complex fermentation procedures;
	Physico-chemical requirements	Extreme agitation or aeration conditions; Large cooling requirement
	Genetic stability	Yield losses; limited process duration
Product	By-product formation	Expensive recovery process
	Stability	Losses in yield, productivity
	Quantity and quality	Costly recovery process; unjustified investments in R & D
Raw material	Availability	Production bottle-neck
	Cost	Economic competitiveness
	Quality/Uniformity	Irregular yields between batches
	Sterilisation requirements	Cost; complex facilities; separate or discontinuous sterilisation
	Stability	Special storage facilities
Equipment	Size and design of fermenter	Scaling up; homogeneity; O <sub>2</sub> distribution and thus fermenter productivity
	Sterilisation systems	Choice of nutrients; cost of sterilisation
	Instrumentation; automation	Degree and quality of control; reliability of the process
Recovery	Various	

## **6.3 Materials And Methods**

General materials and methods used in this chapter have been presented in **Chapter 2**, while those specific to this chapter are presented below.

### **6.3.1 Media, Solutions and Strains**

#### ***Aspergillus* Complete Medium (ACM)**

This medium contains 10 g D-glucose (Sigma, UK), 1 g yeast extract (Oxoid, UK), 2 g Peptone (Oxoid, UK), 0.075 g adenine (Sigma, UK), 1 g Casamino acids (Sigma, UK), 10 ml *Aspergillus* vitamin solution and 20 ml *Aspergillus* salt solution. The pH was adjusted to 6.5 with NaOH and made up to 1L using reverse osmosis water (Paoletti et al., 2005).

#### **250 mM EDTA (Disodium Ethylene Diamine Tetraacetic Acid) pH 8.0**

Exactly 18.61 g of EDTA and 2.0 g of NaOH pellets were placed in a volumetric flask containing 160 ml water and a stirrer. The solution was stirred and allowed to cool to room temperature. The pH was adjusted by dropwise addition of 2 M NaOH solution and the final volume made up to 200 ml with water. The solution was autoclaved before use.

#### **DNA Extraction Buffer**

A buffer for the extraction of fungal DNA was prepared to a final concentration of 250 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA pH 8.0, and 0.5 % sodium dodecyl sulphate pH 7.2 according to Murtagh et al. (1999). The buffer was sterilised by autoclaving before use.

#### **Tris-EDTA (TE) Buffer**

This solution contains 10 mM Tris-HCl and 1 mM EDTA at a final pH of 8.0. One millilitre of 1M tris-HCl at pH 8.0 was added to approximately 80 ml water, followed by 400 µl of 250

mM EDTA at pH 8.0. The solution was then made up to 100 ml with water and autoclaved before use.

### **Tris-acetate-EDTA (TAE) and Tris-borate-EDTA (TBE) Buffers**

These were prepared exactly according to Sambrook et al. (1997).

### **0.2 % Tween 20 agar preparation**

A 10 % tween 20 solution was prepared by the addition of 1ml of tween 20 reagent to water and the solution made up to 10 ml. From this solution, 2 ml was diluted up to 100 ml with water. To this solution, 0.2 g of agar was added and shaken thoroughly to ensure even dispersion of the agar. The solution was then autoclaved.

### **Gel loading buffer**

This was prepared by the mixing 2.4 g sucrose, 15.0 mg xylene cyanol and 15.0 mg of bromophenol blue in 5 ml of water. The solution was shaken thoroughly to mix, then after complete dissolution, final volume was made up to 6 ml with water.

### **Lambda DNA markers for gel electrophoresis**

A set of lambda DNA stocks were prepared for the quantification of DNA by gel electrophoresis. These consisted of 5, 10, 20, 40, 60, and 100 ng markers in 8 µl final volume, prepared by appropriate dilutions of a stock of lambda DNA in TE buffer.

### **DNA ladders**

DNA molecular weight markers or ladders (100 bp and 1 Kb) were prepared for use according to manufacturer's instructions (NEB, UK).

### **Odlum's Oatmeal Agar (OOA)**

Pinhead oatmeal (Odlums Group, Ireland) was used to prepare oatmeal agar medium as follows: 40 g of oats were added to 1 L tap water in a 5L flat-bottomed flask and heated until boiling on a hotplate stirrer, then left to simmer for 45 min. The flask was loosely capped with tinfoil to minimise water loss. The medium was then filtered through two layers of cheese cloth and made up to a final volume of 1 L with tap water. Finally, 20 g of agar was mixed into the solution before final autoclaving at 121°C for 15 min (Robert, 2007). The media were left to cool and 25 ml aseptically dispensed into triple-vented 9 cm Petri dishes.

### **Mixed Cereal Agar (MCA)**

A mixed cereal medium was prepared with the Gerber Multigrain Cereal (New Jersey, USA) meal as follows: Into a 5 L flask, 50 g of multigrain cereal and 15 g of agar were measured and made up to 1 L with tap water. The slurry was then sterilised by autoclaving at 121°C for 15 min. Fifty millilitres were then aseptically dispensed into triple-vented 9 cm Petri dishes.

### **Sorghum Flour Agar (SFA)**

Whole red sorghum flour was used to prepare sorghum flour agar medium. Into a 5 L flask, 50 g of the flour was added. The mixture was stirred thoroughly and the slurry filtered through cheesecloth. Fifteen grams of agar was added and stirred in, and the solution made up to 1 L with tap water and the slurry sterilised by autoclaving at 121°C for 15 min. Fifty millilitres were then aseptically dispensed into triple-vented 9 cm Petri dishes.

### **Itaconic acid solution (60 g/l)**

This was prepared by dissolving 30 g of itaconic acid in 400 ml of water and mixing well. This solution was then made up to 500 ml and sterilised by autoclaving.



### Itaconic acid Potato Dextrose Agar (IAPDA)

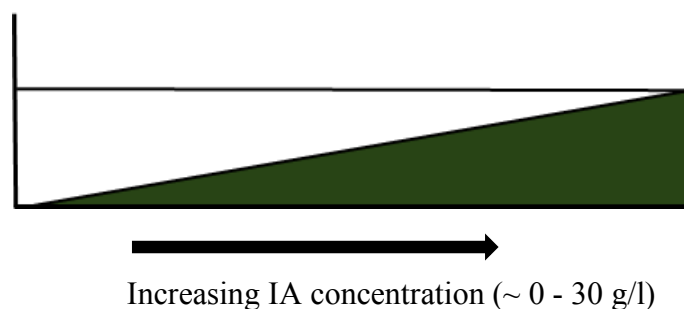
Double strength PDA (DSPDA) was first prepared by the addition of 78 g of PDA to a 1 L Duran bottle and made up to a litre with water. This solution was heated to ensure dissolution and mixing of the powder, then sterilised by autoclaving. The sterile DSPDA was then mixed with water and 30 g/l itaconic acid solution in various proportions to obtain various concentrations of IAPDA media (**Table 6.2**). The IA was not added to the PDA prior to autoclaving as the resultant low pH would have prevented the medium gelling after autoclaving. However, this was averted by sterilising the components separately and mixing just before pouring. The media were dispensed in 25 ml aliquots into 9 cm Petri dishes and labelled. These plates were used in the IA tolerance test.

**Table 6.2:** Preparation of various concentrations of itaconic acid potato dextrose agar (IAPDA) plates

<b>WATER (ml)</b>	<b>ITACONIC ACID SOLUTION*(ml)</b>	<b>DS<sup>†</sup> PDA (ml)</b>	<b>IAPDA FINAL CONCENTRATION (g/l)</b>
0.00	50.00	50.00	30
16.67	33.33	50	20
33.33	16.67	50	10
41.67	8.33	50	5
45.00	5.00	50	3

\* The itaconic acid solution has a concentration of 30 g/l; † DS = Double Strength

In addition, rectangular 9 x 12 x 3 cm IAPDA concentration-gradient agar plates were also prepared as previously described by Yahiro et al. (1995) for the efficient selection of *A. terreus* isolates with increased IA production. Two layers of medium were created in the plates by elevating one half slightly and pouring 100 ml of 30 g/l IAPDA into it. Care was taken to avoid disturbing it until the medium set in this way with the IAPDA tailing off at one end of the plate. Next, the plate was placed flat on the bench and 200 ml of regular PDA at 65°C was poured into it starting from the emptier end. This technique resulted in a plate containing a uniformly increasing itaconic acid concentration from left to right (**Figure 6.4**).



**Figure 6.4:** Itaconic acid concentration-gradient plate: the dark green lower layer indicates the 30 g/l IAPDA and the top white layer, regular PDA.

### *Aspergillus terreus* Isolates

The isolates employed in the sexual crossing were 49-1, 49-5, 49-22, 49-40, 49-43, 49-44 and 49-45. They were selected on the bases of either proven sexual fertility (Arabatzis and Velegraki, 2012) or itaconic acid yield (the present study). Strains 49-1, 49-5, 49-22, 49-43 and 49-45 were selected for their high itaconic acid production capacities (**Section 4.3.2.3**) while 49-40 (*MAT1-1*) and 49-44 (*MAT1-2*) were previously reported as prolific maters (Arabatzis and Velegraki, 2012).

## 6.3.2 Methods

### 6.3.3 Mutagenesis

The strains which exhibited highest levels of itaconic acid fermentation on RBDAH, namely 49-5 and 49-22 (**Section 4.3.2.3**), were exposed to ultraviolet irradiation in order to induce mutations in their DNA that could potentially improve their itaconic acid production performance. This work involved multiple approaches as described below.

#### 6.3.3.1 Tolerance of *Aspergillus terreus* Isolates on IAPDA Plates

This was performed in order to determine the maximum itaconic acid concentration that native strains 49-5 and 49-22 can tolerate on solid media. Exactly 25  $\mu$ l of a  $1 \times 10^6$  spore/ml suspension of each strain was plated on 9 cm plates of IAPDA in replicates of six plates per strain. The plates were incubated for a week, but observed daily for growth. Colonies

observed were counted and recorded and the highest concentrations of IAPDA at which the strains grew were noted.

#### **6.3.3.2 Kill Curve for UV Irradiation**

A spore suspension containing  $1 \times 10^5$  spores/ml of 49-5 was prepared in sterile 0.05 % tween agar solution. The suspension was aseptically dispensed in 4 ml aliquots into sterile 9 cm Petri dishes containing a sterilised stir bar. The interior of the UV chamber was wiped clean with 70 % ethanol, and the UV lamp adjusted to 35 cm away from the dish then turned on for 10 minutes to sterilise the environment. The lamp was turned off, the suspension placed on the magnetic stirrer plate and the Petri dish lid removed. The stirrer was turned on to ensure even mixing of the suspension. An aliquot of 0.2 ml was collected (time zero control), the hood closed and the lamp turned on. Samples were collected at regular intervals up to four minutes then the lamp was switched off. The same process was repeated for strain 49-22. Next, 50  $\mu$ l aliquots of samples collected at each time point was plated in triplicates on PDA plates and incubated at 37°C for five days. The plates were counted after 3 days and the number of colonies totalled and expressed as a percentage of the control. A kill-curve was plotted and the 5 % survival point determined (Ashour, 2014).

#### **6.3.3.3 Ultraviolet Irradiation and Prescreening**

A UV irradiation experiment was performed in an attempt to generate mutant *A. terreus* strains with improved IA fermentation characteristics. A suspension with  $3 \times 10^6$  spores/ml for both 49-5 and 49-22 was exposed to UV light and the irradiation ended at the 5 % survival point previously determined. In a rationalised screening process (**Section 6.1.1**), the exposed spores were then plated in triplicates on the concentration-gradient IAPDA plates that had a section of the gradient containing the previously determined itaconic acid concentration to which the strains showed maximum tolerance. The plates were incubated upside down for a total of four days after which a total of 250 mutant progeny, 90 of 49-5 and 160 of 49-22, were selected by hyphal tipping or single spore isolation techniques. Selection was commenced from the high concentration ends of the gradient plates (i.e. those mutants showing highest IA resistance), with emerging mutants transferred to PDA slants and incubated for five days.

#### **6.3.3.4 Mutant Fermentations**

The mutants selected from the IAPDA were fermented in the defined glucose medium of (Kuenz *et al.*, 2012) in bespoke 12-well boxes with 30 ml volume wells, which had the advantage of allowing for higher-throughput fermentations than shake flasks. Spore suspensions were prepared from the mutants (**Section 2.2.4.1**) and used in 10 ml fermentations. After these screening fermentations, the highest-yielding mutants were then employed in shake-flask fermentations to verify yields. Samples were collected randomly from about 20 wells after 3 days to check for itaconic acid levels and residual glucose content. The fermentation were left to proceed until considerable glucose depletion was observed. They were then terminated on Day 7 and analysed for itaconic acid as described previously (**Section 2.2.6**).

#### **6.3.4 Sexual Crossing**

This section describes protocols applied in an attempt to induce sexual reproduction in the *Aspergillus terreus* strains and isolate recombinant progeny.

##### **6.3.4.1 Culture Preparation For DNA Extraction**

To obtain genomic DNA from the various strains of *A. terreus* species, mycelia were produced in liquid cultures. A spore suspension was prepared by washing agar slants with 0.05% (v/v) tween 80 solution and gently agitating the surface of the slant with a sterile cotton swab. The suspension was then transferred to 250 ml conical flasks containing 50 ml of ACM broth. The cultures were incubated at 28°C on a rotary shaker at 150 rpm for 3 days. The mycelia were then collected by filtration through a sterile folded square of Miracloth<sup>®</sup> (Calbiochem, USA) and rinsed thoroughly and excess medium squeezed out. The fungal tissue was freeze-dried and DNA extracted.

##### **6.3.4.2 DNA Extraction**

High quality DNA was extracted from freeze-dried mycelia according to the method of Murtagh *et al.* (1999) with a slight modification. The freeze-dried tissue was ground to a fine

powder under liquid nitrogen using a ceramic mortar and pestle. Approximately 500 mg was suspended in 1 ml of DNA extraction buffer in sterile 2 ml centrifuge tubes. The tubes were inverted several times to mix then incubated at 65°C for 30 minutes and frequently mixed by inversion during this period. The tubes were then centrifuged at 13,000 rpm for 10 min and in a modification of the method of Murtagh et al. (1999), 600µl of the supernatant was transferred from each tube into a 2 ml phase lock gel (PLG) tube (by the company 5Prime).

Next, 600µl of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) was added to each tube, inverted several times to mix and then centrifuged at 13,000 rpm for 10 min. The phenol:chloroform:isoamyl alcohol step was repeated once to ensure complete removal of proteins. Next, the upper aqueous phase was transferred to a new 1.5 ml tube and 0.7 volumes of isopropanol added. The tubes were invert several times and incubate at -20°C for at least 30 min. They were then centrifuged at 13,000 rpm for 10 min and the supernatant discarded. The pellet was washed with 700µl of 70% ethanol, centrifuged at 13,000 rpm for 10 min and ethanol completely removed by pipetting. The tubes were left to dry in a laminar airflow cabinet for 5-10 min before re-suspending the pellet in 100 µl of TE buffer overnight. The DNA concentration was measured using a Nanodrop® ND-1000 (Nanodrop Wilmington, USA) spectrophotometer.

The integrity and yield of DNA extractions was verified by electrophoresis in 1.2% (w/v) agarose gels with reference to lambda DNA standards. One microliter of ethidium bromide solution (0.5 µg/ml) was added to the anodic end of the tank and the set-up run at 100mV for 1 hour after which bands in the gel were visualised via UV light and photographed using a Chemidoc XRS transilluminator (Bio-Rad Laboratories, UK).

#### **6.3.4.3 Species Verification by PCR**

The various *Aspergillus terreus* isolates (**Appendix 1**) were identified by molecular means to confirm species identity and eliminate cryptic species. A region of the gene encoding beta-tubulin was amplified using previously reported *benA* primers (Balajee *et al.*, 2009) while a region of the calmodulin gene (*calM*) was amplified using degenerate primers CF1 and CF4 shown in **Table 6.3** [Peterson et al., 2005 cited in Balajee et al. (2009)]. The PCRs were conducted in 50 µL reactions each containing 31.5 µL of DNase free water 10µL Phusion®

High Fidelity buffer, 1 µL of 10mM dNTPs, 2.5 µL each of 10mM forward and reverse primers, 0.5 µl of Phusion® DNA Polymerase and 2 µl of ~20ng genomic DNA template. The amplifications were carried out in a Techne Genius thermal cycler (Techne Ltd, UK) with the following cycle parameters: an initial denaturation step at 98°C for 2 min; followed by 35 cycles of denaturation at 98°C for 20s, 58.8°C for 30s and elongation at 72°C for 20s with a final elongation at 72°C for 3 min. All steps were performed at the maximum ramp rate.

**Table 6.3:** Amplification primers used in the species verification analysis for isolates of *Aspergillus terreus*.

PRIMER NAME	SEQUENCE 5' – 3'
benA F	GGGGATAGGATGTTTTGTGACA
benA R	GGTCAACGAGGACGGCACGA
CF1 F	GCCGACTCTTTGACYGARGAR
CF4 R	TTTYTGCATCATRAGYTGGAC

#### 6.3.4.4 PCR Product Purification And Verification

To verify that the *benA* and CF1 and CF2 amplicons were of the desired sizes of about 300bp and 700bp respectively, a portion of the PCR product (Section 4.2.4) was checked by electrophoresis as described in section 4.2.3 with reference to 100bp DNA ladder, while the remaining product was purified with a NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The concentration of the resulting product was then determined with the Nanodrop machine and sequenced at the DNA sequencing facility of the University of Nottingham Medical School. The sequences obtained were analysed with MacVector 11 software (Macvector, USA). The sequences were then searched for online and compared with published sequences in the Basic Local Alignment Search Tool (BLAST) database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm the target identity. Sequences were also aligned using the Multiple Sequence

Alignment tool on [www.tcoffee.vital-it.ch](http://www.tcoffee.vital-it.ch), and ClustalW alignment on [www.ebi.ac.uk/Tools/msa/clustalw2](http://www.ebi.ac.uk/Tools/msa/clustalw2).

#### 6.3.4.5 Mating-Type Determination

A mating-type assay was conducted on the verified *A. terreus* isolates by PCR amplification of the *MAT1-1* and *MAT1-2* genes according to (Eagle, 2009) using primers which detected the presence of either the alpha-domain encoding sequence in *MAT1-1* isolates or the HMG-domain encoding gene in *MAT1-2* isolates (**Table 6.4**). The PCRs to determine mating type were performed using Phusion® High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5 µl of 5x HF Buffer, 0.5 µl of dNTPs mix (comprising 10mM each), 1.25µl each of 10µM forward and reverse *MAT* primers, 0.25µl of Phusion® DNA polymerase and 15.75 µl sterile H<sub>2</sub>O. Reactions were prepared in an ice bucket as master mixes which were then aliquoted into individual PCR tubes before the addition of 1µl of the DNA template. The amplification of the fragments was performed on a Techne Genius thermal cycler with the following cycle parameters: an initial denaturation step of 98°C for 2 min followed by 35 amplification cycles of 98°C for 20 s, 65.1°C for 30 s and 72°C for 20 s, and a final extension at 72°C for 5 min (all at the maximum ramp rate). The products were held at a final storage temperature of 4°C before analysis by agarose gel electrophoresis.

**Table 6.4:** Amplification primers for mating-type determination in the *MAT* region of *Aspergillus terreus*

PRIMER NAME	SEQUENCE 5' – 3'
AteM1F	GCGAGGCAGACACATTCAGGAT
AteM1R	CGAGGATGCCAATAAAACCAGC
AteM2F	TCTATCGCCAGCACCATCATCC
AteM2R	CTTGTTGTGGTGGTGGTCGTTCT

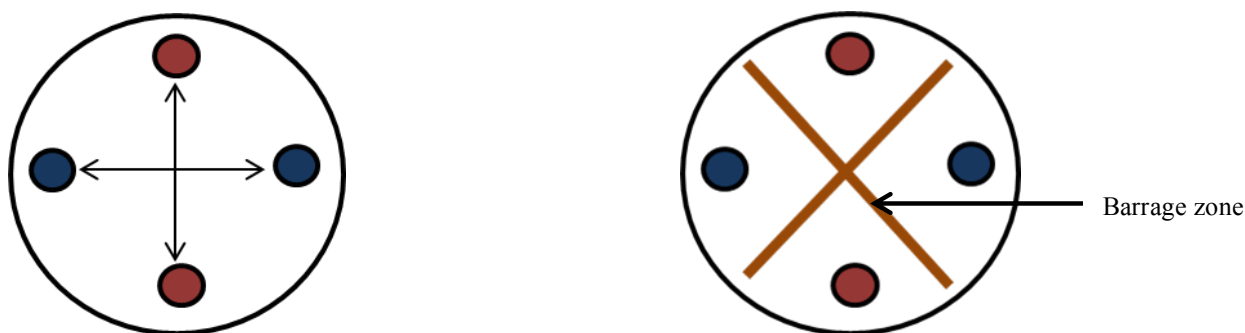
#### **6.3.4.7 Spore Suspension Preparation**

Spore suspensions of conidiospores for the sexual crossing were prepared from PDA slants that were less than six weeks old by gently rubbing a sterile cotton swab on the surface of the slant. The swab was then transferred to 5 ml of 0.05 % Tween80 solution in a universal tube and swirled to remove spores. The suspension was vortexed to break clumps of spores and ensure thorough mixing, diluted and counted with a Neubauer haemocytometer. The suspension was then adjusted to a final concentration of  $5 \times 10^5$  spores per ml with a solution of 0.05% Tween 80 with 0.2 % agar and used immediately in crosses.

#### **6.3.4.8 Crossing Protocol**

Sexual crosses were set up in duplicate between selected *MAT1-1* and *MAT1-2* isolates [determined from the earlier *MAT* diagnostic PCRs, and selected for known sexual fertility according to Arabatzis and Velegraki (2012) and high IA yield in the present study] in all possible combinations on 9 cm Petri dishes containing oatmeal agar (OA), mixed cereal agar (MCA) or sorghum flour agar (SFA) according to O’Gorman et al. (2009) and Arabatzis and Velegraki (2012). Exactly 50 µl of the spore suspension of one mating type (described in **Section 4.2.7.1**) was aliquoted onto two spots on the surface of the medium 4-5 cm apart and parallel to each other, and the same performed for the opposite mating time, perpendicular to the first two spots as illustrated in **Figure 6.5**. The plates were left to dry for 30 min in a Class II Biosafety cabinet, covered and incubated upside down. For each combination, one pair was sealed with Nescofilm, and another pair was left unsealed but duplicates were stacked in clean clear plastic bags (3 pairs per bag) and incubated upside down at 32°C. A similar set of 2 pairs of crosses was prepared, sealed or packed in plastic bags and incubated at 37°C in stacks of 2 dishes per layer. All crosses were incubated for 12 weeks. The schedule of the crosses set up can be found in **Appendix 5**. Crosses were finally inspected for formation of hyphal aggregations possibly correlated with sexual reproduction, and if present the diameters of hyphal aggregations were measured using a stereomicroscope fitted with a graticule. Images were taken with a Canon microscope fitted to a computer.





**Figure 6.5:** *Left:* Spheres of different colours represent opposite mating types; arrows indicate distance of 4-5 cm between aliquots of the spore suspensions. *Right:* A barrage formation that resulted at the point of interaction after several weeks of incubation.

#### 6.3.4.9 Evidence of Genetic Recombination

##### 6.3.4.9.1 Microscopy

Upon maturity, hyphal masses from several crosses were removed and attempts made to collect ascospores as described by Swilaiman (2013). A large hyphal mass was removed at the barrage zone and transferred to a plate of 4% tap water agar (TWA). The mass was gently crushed under a Nikon SMZ-2B dissecting microscope at a 40 x magnification with a flame sterilised wire pin to release spherical putative cleistothecia. A number of putative cleistothecia were carefully rolled away from the hyphal debris with the pin, and continuously rolled over the surface of the plate to remove adhering conidiospores until they left negligible or no trail of spores behind. One was then placed on a glass slide and a drop of water added to it and covered with a cover slip and observed microscopically. The slip was then pressed gently to break the cleistothecia and carefully release its contents. The possible presence of asci and ascospores was then investigated as evidenced by genetic recombination.

##### 6.3.4.9.2 Single Ascospore Cultures

Four methods were used to try and produce pure cultures of ascospores, if present. Firstly, at least 10 putative cleistothecia were collected as described above by rolling on TWA. They were then transferred to 2 ml Eppendorf tubes and 100  $\mu$ L of 0.05% Tween 80 solution added and then crushed with a sterile pin to release ascospores. The suspension was made up to 500  $\mu$ L with tween solution and any material adhering to the sides of the tube washed down. The suspension was vortexed vigorously and 100  $\mu$ L plated out onto PDA plates and incubated at 37 °C overnight. In the second method, it was considered possible that the

ascospores might be more heat tolerant than conidia, so after transferring the cleistothecia to the tubes and 100µl of 0.05% Tween 80 solution added, the suspension was heated at 65°C for 10 min based on the method of Swilaiman (2013) to kill any remaining conidia, before being crushed with a sterile pin to release ascospores. The suspension was then treated as above and incubated at 37 °C overnight. A third method was attempted which involved inactivating the contaminating conidiospores surrounding the cleistothecia using alcohol. To the surface of TWA plates, one millilitre of 70 % ethanol was added and the cleistothecia rolled over the surface to remove adhering conidia and debris for no longer than a minute to minimise the risk of killing any ascospores in asci within cleistothecia. The cleistothecia were then transferred to fresh TWA plates, rolled again and finally transferred to a tube containing 100 µl of 0.05% Tween 80 solution and treated as above. Lastly, the cleistothecia were cleaned by rolling on TWA, were then removed using forceps, dissected with a platinum wire pin under the stereomicroscope and the contents scooped out and transferred to a 0.05% Tween 80 solution. This solution was mixed and plated and incubated as described above.

After overnight incubation, single germinating colonies were removed with a platinum wire loop under a Nikon stereomicroscope and transferred to PDA slants in triplicates to produce single cultures of putative ascospores. They were incubated for 4 days and liquid cultures prepared as described in **Section 6.3.4.1** and DNA extracted as described in **Section 6.3.4.2**.

#### 6.3.4.9.3 RAPD-PCR ANALYSIS

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis was carried out on all the parental strains employed in the crosses and on the putative ascospore progeny in order to determine clonality and assess possible genetic recombination. The primers employed are shown in **Table 6.5**. RAPD-PCR was performed according to Murtagh et al. (1999) in 25 µl reactions that contained 2.5 µl 10x Buffer, (containing 1.5mM MgCl<sub>2</sub>), 0.375 µl dNTPs (10 mM each), 0.1 µl of a single 8-16 base-pair (50µM) RAPD primer (**Table 6.5**), 0.375 µl of DyNAzyme II (2U/µl) DNA Polymerase (Finnzymes, Finland), 1 µl of 10 ng genomic DNA, and 20.65 µl ultra-pure water. Blank tubes were also set up in each run which contained similar reagents but with ultra-pure water replacing the DNA template.

Fragment amplification was performed on a Techno Genius thermal cycler at the conditions described by Murtagh et al. (1999) comprising 45 cycles of denaturation at 93 °C for 30 s,

annealing at 37 °C for 40 s and elongation at 72 °C for 80 s, followed by a final elongation stage of 72 °C for 5 min after which the products were held at 4 °C. Exactly 6.25 ml of loading buffer was then added to the amplification products and 15 µl of this mix analysed by electrophoresis in 1.3 % (w/v) agarose gels placed in TBE buffer with the application of 150 V current for approximately 3 hours and sized using a 100 bp DNA ladder. The gels were then stained by carefully transferring them into a glass tank containing a solution of 2 mg of ethidium bromide in 200ml of TBE buffer, and rocking it for 10 minutes. The bands were visualised via UV light and photographed using a BioRad Chemidoc XRS (BioRad Laboratories, UK) transilluminator.

**Table 6.5:** List of primers used in RAPD-PCR for the investigation of genetic recombination.

PRIMER NAME*	SEQUENCE 5' – 3'
AM III	ACGTCCCACT
AM IV	GATAGATAGATAGATA
R108	GTATTGCCCT
RCO8	GGATGTCTGAA
CII	GCGCACGG

\* These primers had been previously selected from an initial screening of approximately 10 RAPD primers based on their ability to produce polymorphic bands between the parental isolates (data not shown).

### 6.3.5 Fermentation Optimisation

In **Chapter 4**, it was demonstrated that itaconic acid can indeed be produced by *A. terreus* from sorghum bran dilute acid hydrolysates. The fermentation factors/variables of temperature, agitation speed, pH and inoculum size employed so far were obtained from literature and were assumed to be suitable for this medium-strain system, although these parameters were based on use of a defined glucose medium. Fermentation conditions can have a profound impact on the yields obtained so there was considered to be a need to optimise conditions for the selected strain and for use of RBDAH because of the potential complexity and peculiarity of interaction in every medium-strain system. Therefore an RSM

approach was adopted to try to optimise fermentation factors to thereby increase the yield of itaconic acid.

There are three stages in a RSM optimisation technique. Firstly, the parameters with the greatest impact on the fermentation process are identified by screening experiments, with full or fractional factorial designs being used for this purpose. In addition, these experiments will give information about the direction of improvements due to varying the parameter, and the suitable levels of the parameter. As some factors will be eliminated due to minimal impact on the fermentation, the more significant ones are retained and examined in greater depth (de Cássia Lacerda Brambilla Rodrigues et al., 2012). Next, a RSM method is selected and the model equation's prediction is verified. Lastly, response surface plot and contour plots are obtained as functions of the independent parameters, and the optimum points determined (de Cássia Lacerda Brambilla Rodrigues et al., 2012). This was the pattern followed in the next section for the optimisation of the itaconic acid fermentation on RBDAH.

#### ***6.3.5.1 Half-Factorial Screening of Factors***

Various factors are known to influence itaconic acid fermentation yields including pH, temperature, glucose and mineral content, agitation and fermentation duration (Ahmed El-Imam and Du, 2014). A screening experiment was performed to determine which factors contribute most significantly in the yields when using RBDAH. This was facilitated by the fact that since RBDAH is not a defined medium, medium composition relating to glucose and mineral content was effectively fixed, so these factors did not need to be screened for or optimised. Similarly, the optimum fermentation duration has already been determined in the time-course experiments performed in **Chapter 4**, and it was thus not investigated as a factor.

Therefore a half-factorial screening experiment was performed investigating the effect of pH of RBDAH, quantity of *A. terreus* inoculum, agitation speeds (which indicates aeration) and incubation temperature on itaconic acid concentration after a 4-day fermentation. The ranges of the factors are shown in **Table 6.6** below. The half-factorial screening design ( $2^{4-1}$ ) was created with the Statgraphics software in two blocks of nine experiments with one centrepoin t each; observing for three responses, itaconic acid, residual glucose and mycelial mass. The design was aimed at studying the effects of the factors in 18 runs that were fully randomised to protect against the effects of lurking variables, and is presented in **Table 6.7** below.

**Table 6.6:** Variables and the levels employed in a half-factorial design for itaconic acid fermentation.

FACTOR	CODE LEVEL		
	-1	0	1
	ACTUAL LEVEL		
TEMPERATURE (°C)	33	35	37
AGITATION (rpm)	180	200	220
pH	2.7	3.1	3.5
INOCULUM SIZE (log)	5.48	5.813	6.00

**Table 6.7:** Half factorial design used to screen factors for itaconic acid fermentation using *Aspergillus terreus*.

Block	Temperature (°C)	Agitation (rpm)	pH	Inoculum size (Log <sub>10</sub> )
1	33	180	2.7	5.48
1	33	180	3.5	6.0
2	33	180	2.7	5.48
2	33	180	3.5	6.0
1	37	180	3.5	5.48
1	37	180	2.7	6.0
2	37	180	3.5	5.48
2	37	180	2.7	6.0
1	35	200	3.1	5.813
2	35	200	3.1	5.813
1	33	220	2.7	5.48
1	33	220	3.5	6.0
2	33	220	2.7	5.48
2	33	220	3.5	6.0
1	37	220	3.5	5.48
1	37	220	2.7	6.0
2	37	220	3.5	5.48
2	37	220	2.7	6.0

#### 6.3.5.2 Factor Optimisation by Response Surface Methodology

The three selected factors were optimised using response surface methodology because it was believed that the various factors and their interactions would have an effect on the desired response. The experimental design chosen was known as the central composite design (CCD) and RSM uses this design to fit a model by least squares technique. It is the most commonly used RSM design (support.minitab.com). They are full or fractional designs with centrepoints, in addition to axial points that allow for the estimation of curvature. The adequacy of this model is verified by ANOVA while the response surface plot generated can be employed to decipher the optimum conditions for itaconic acid production.

In this section, a CCD in the form of a  $2^3 + \text{Star}$  design was created which studied the effects of 3 factors in 32 runs. The design was run in 2 orthogonal blocks to allow model terms and block effects to be estimated independently and minimise variation in the regression coefficients. The design also had two centrepoin ts per block with the order of the experiments fully randomized to protect against the effects of lurking variables. It was completely rotatable to provide constant prediction variance at all equidistant points from the centre of the design. The base design is presented below (**Table 6.8**). The set of conditions which give the highest itaconic acid yields were then selected as optimum.

**Table 6.8:** Base design for the central composite design employed for the optimisation of the itaconic acid fermentation in RBDAH by *Aspergillus terreus*.

Factors	Low	High	Units	Continuous	Responses	Units
Temperature	30.0	40.0	C	Yes	Itaconic acid	g/l
pH	2.0	4.0		Yes	Residual glucose	g/l
Inoculum	5.0	6.0	Log <sub>10</sub>	Yes	Mycelial mass	g

### 6.3.6 Optimisation Verification and Scale-Up

The optimised fermentation was repeated a total of 3 times to establish that any change in yields observed were not due to experimental error. Next the process was scaled up from 25 ml fermentations to 200 ml and 500 ml fermentations in 2 L and 5 L volumetric flasks, respectively.

## 6.4 Results

Three methods were attempted to improve the itaconic acid yields from red sorghum bran dilute acid fermentation by *Aspergillus terreus*, namely UV mutagenesis, genome-wide recombination by sexual crossing and response surface methodology for the optimisation of the fermentation process. Findings from these investigations are presented below.

### 6.4.1 Ultraviolet Mutagenesis

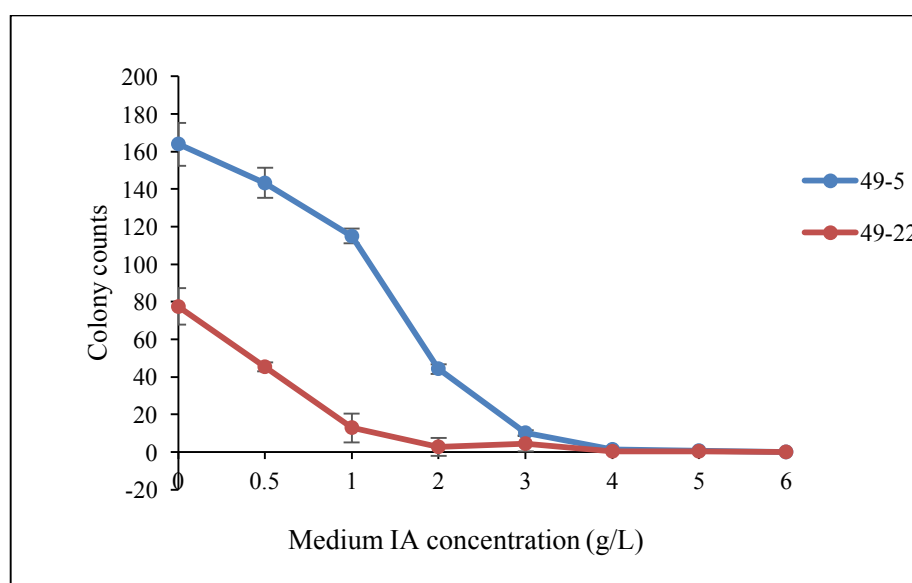
Isolates 49-5 and 49-22 were irradiated with UV light to create mutants that would hopefully produce *A. terreus* strains with higher levels of itaconic acid than the parents.

#### 6.4.1.1 Tolerance of *Aspergillus terreus* on IAPDA

The two strains employed, 49-5 and 49-22, were tested on IAPDA plates containing various amounts of itaconic acid to determine their tolerance threshold to allow a rationalised selection process for highly itaconic acid tolerant mutants.

It was observed that the maximum IA concentration that the strains could tolerate in the IAPDA medium was about 4.0 g/l after which the *A. terreus* did not grow (**Figure 6.6**). Strain 49-5 had consistently higher colony counts than 49-22, even on control medium, at a statistically significant level (data not shown).

This data about the minimum inhibitory concentration was used to inform the IA levels chosen for later screening plates, which were therefore prepared in a way to contain concentrations higher and lower than the 4.0 g/l IA along the gradient (**Figure 6.4**).

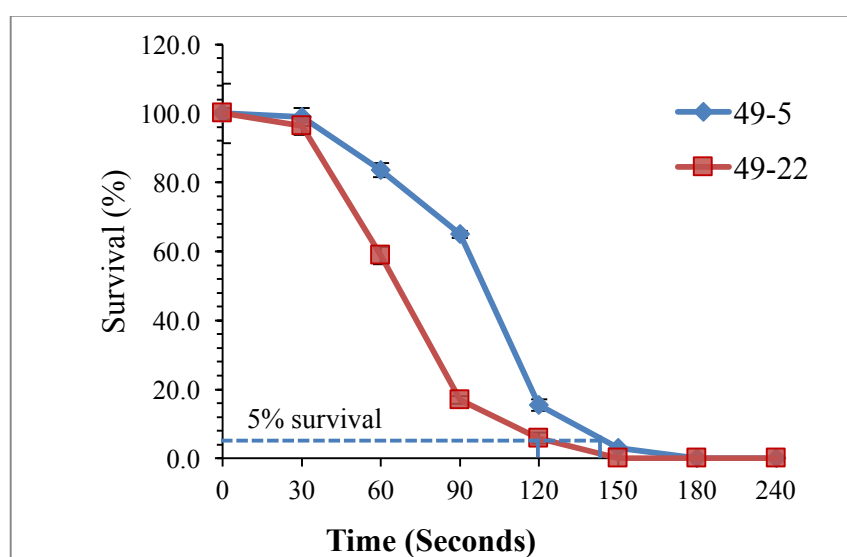




**Figure 6.6:** Itaconic acid tolerance in two strains of *Aspergillus terreus* grown on PDA plates with varying concentration of itaconic acid. Values are averages of 6 measurements and error bars represent standard deviations.

#### 6.4.1.2 UV Irradiation Kill Curve

Isolates 49-5 and 49-22 were exposed to UV irradiation for various times and survival measured after five days incubation (Section 6.3.3.2). It was found that the 5 % survival times were different for the strains, with 49-5 attaining it at about 145 sec and 49-22 at 120 sec (Figure 6.7) and according to t-tests these differences were not statistically significant ( $t = 1.003$ ;  $df = 16$ ;  $P = 0.3211$ ), implying that overall, they were killed by the irradiation at similar rates with the 49-22 being more sensitive.

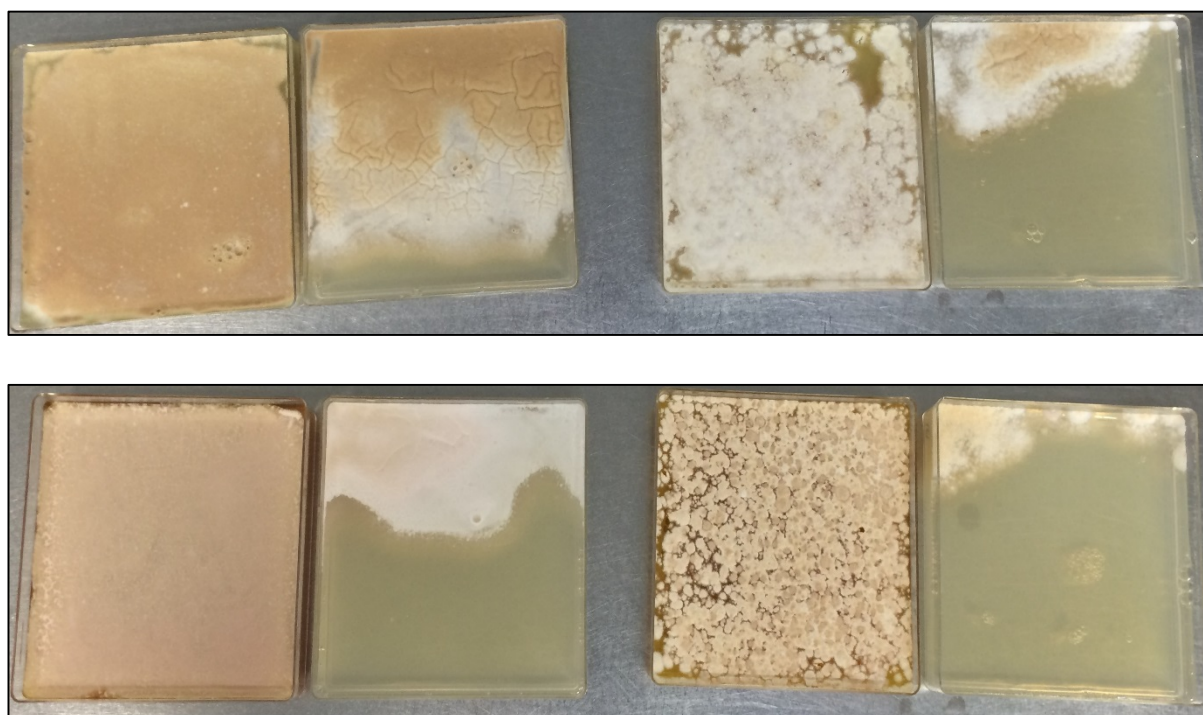


**Figure 6.7:** Kill curve showing the decline in population survival of two *Aspergillus terreus* isolates due to exposure to ultraviolet irradiation. The broken lines show the 5 % survival points for both strains. Values are averages of triplicate measurements and error bars represent standard deviations.

#### 6.4.1.3 UV Irradiation and Prescreening

Spore suspensions of strains 49-5 and 49-22 were exposed to the UV light for 120 sec and 135 sec respectively and then plated on IAPDA plates in duplicates and incubated (Day 0). After two days, it was observed that the colonies were starting to grow at the lower

concentration end of the plate and the growth moved outward along the gradient over time. It can be seen in **Figure 6.8** below that the mutant cultures were less numerous than the parent cultures and the growth of parental and mutant cultures of both strains on IAPDA was less vigorous than on the PDA plates shown for comparison.



**Figure 6.8:** Cultures of parent and mutant progenies of *Aspergillus terreus* 49-5 and 49-22 on control PDA and 30 g/l IAPDA plates. *Top - Aspergillus terreus* 49-5, *Left to Right*: Parental strain on PDA; on IAPDA; Mutant 49-5 progeny on PDA; on IAPDA. *Bottom – Aspergillus terreus* 49-22, *Left to Right* Parental strain on PDA; on IAPDA; Mutant 49-22 progeny on PDA; on IAPDA. IA concentration increasing from top to bottom of plates.

Spores or hyphal tips were thus picked from the higher end of the gradient on Day 3, and newly germinating colonies picked on Day 4. These were assumed to show a higher tolerance for IA and then subjected to fermentation in RBDAH.

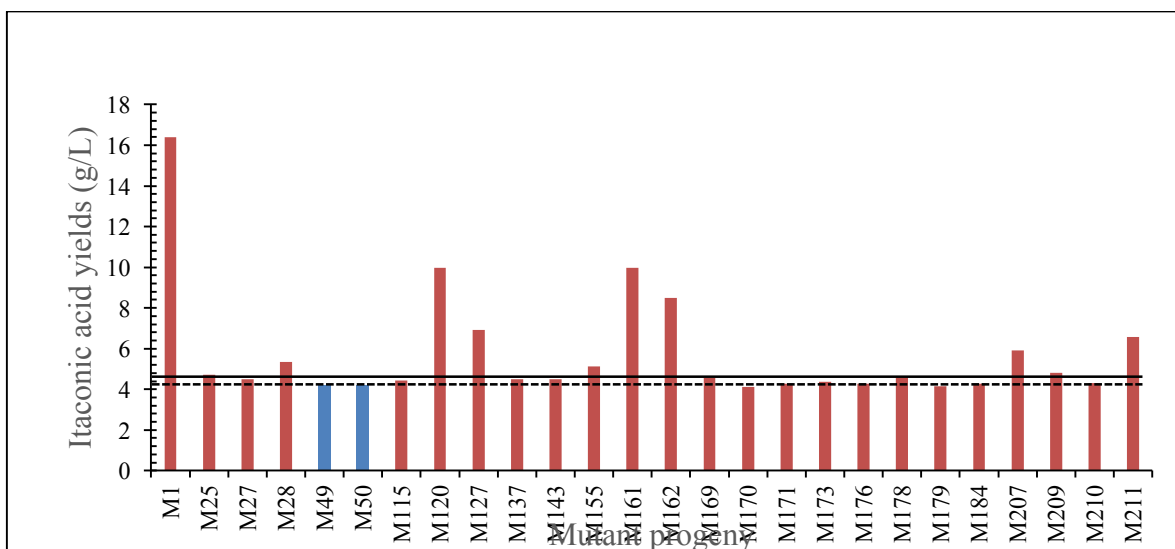
#### 6.4.1.4 Mutant Itaconic Acid Fermentation

A total of 90 and 160 UV mutants were selected from mutagenesis of isolates 49-5 and 49-22, respectively. All 250 mutants were employed in RBDAH fermentation in custom boxes which allowed considerably higher throughput than shake flasks (**Figure 6.9**), and analysed for itaconic acid. By Day 3 of the fermentation, the yields in a random sample were relatively low (results not shown) so all fermentations were first sampled on Day 4, before a final sampling on Day 7 when fermentations were terminated.

It was found that the yields of almost all mutants were lower than those of the parents at Day 4 and the residual glucose content remained high at this point (results not shown). However, by Day 7 the glucose had mostly been exhausted and at this point some UV mutants showed higher itaconic acid production than the parental isolates (**Figure 6.10**). The parental strains, 49-5 and 49-22, produced 4.3 g/l and 4.7 g/l IA, respectively, whilst the highest-producing mutant strain was M1 which showed a 3.5-fold increase over the 49-22 parent (at 16.4 g/l), while M161 and M162 showed 2.1- and 1.8-fold increases, respectively.

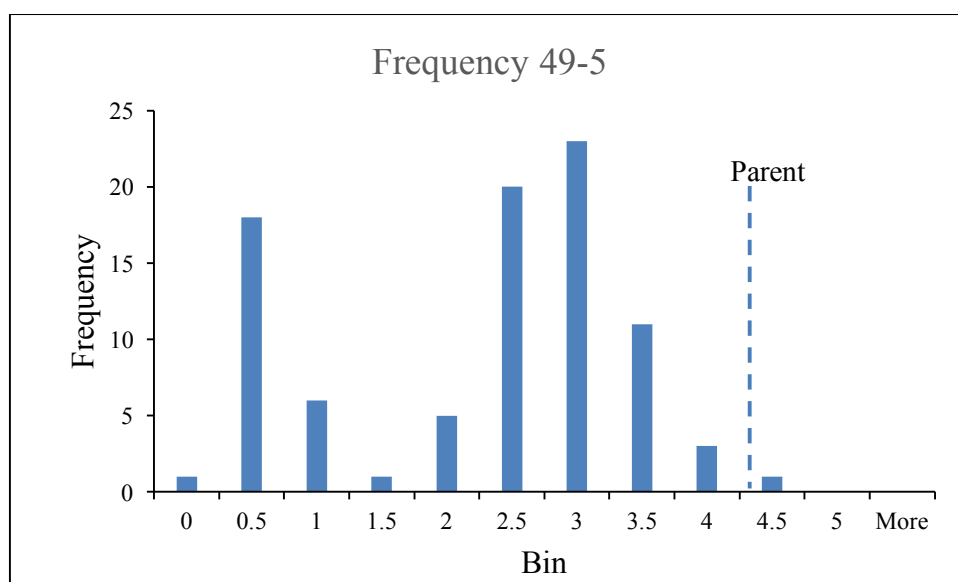


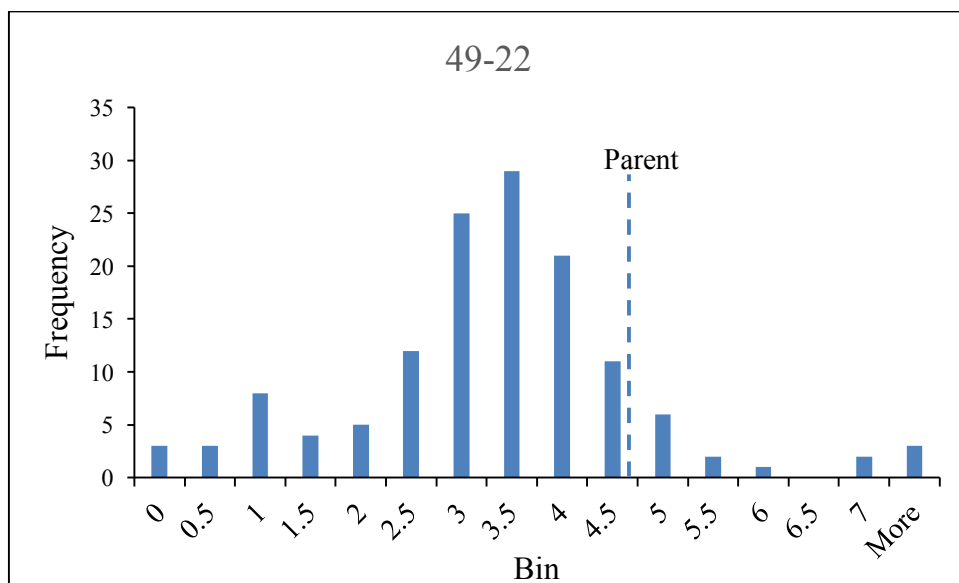
**Figure 6.9:** A custom 12-well box showing the appearance of M59-M70 at the end of the mutation screening fermentation. The white rings inside the wells are *Aspergillus terreus* growth.



**Figure 6.10:** Itaconic acid concentrations of highest-yielding mutants obtained from UV mutagenesis of two isolates of *Aspergillus terreus* after fermentation for seven days. Blue bars represent 49-5 mutant progeny, and red bars 49-22 mutant progeny; dashed line represents parent 49-5 yields (4.3 g/l) while continuous line represents parent 49-22 yields (4.7 g/l).

In total eight mutant offspring were found to surpass the parent 49-22 IA output, whereas no mutants of 49-5 could be detected that showed higher IA production than the parent strain (Appendix 6).





**Figure 6.11:** Frequency histograms of distribution of itaconic acid yields by *Aspergillus terreus* UV mutants in RBDAAH fermentation. Top: UV mutants derived from 49-5, n = 90; Bottom: UV mutants derived from 49-22, n=160. Bins separate IA yields into classes 0.5 g/L apart; Frequency shows number of progeny in each bin.

A histogram plot of the yields from all mutant progeny showed that the yields, especially for 49-22, followed a near normal distribution for both strains (**Figure 6.11**). However, when the 14 highest-yielding mutants (including M1, M161 and M162) were selected and the fermentations repeated in regular shake flasks, this disappointingly resulted in a low overall performance with none of the strains producing yields as high as the parents (**Table 6.9**). Considering that these experiments were performed in triplicate, it was considered unlikely that there was experimental error. Mutant M207 was the highest yielder with 5.12 g/l of itaconic acid, but which still fell short of the yield of the parent 49-22 at 7.18 g/l. Since the mutations yielded no new highly-yielding strains, other follow up processes such as enhancer screens were not performed.

**Table 6.9:** Itaconic acid yields after 7 days fermentation from shake-flask experiments with high-yielding mutants of *Aspergillus terreus* obtained from UV screening experiments.

<i>Aspergillus terreus</i> strain	ITACONIC ACID (g/l)	<i>Aspergillus terreus</i> strain	ITACONIC ACID (g/l)
PARENT 49-22	7.18	M51	0.00
PARENT 49-5	5.71	M52	0.00
M1	3.10	M111	0.00
M2	0.85	M112	1.00
M4	1.93	M113	1.58
M7	3.20	M114	1.37
M8	1.58	M115	2.47
M12	3.07	M116	2.20
M14	2.78	M120	2.42
M17	1.30	M127	3.64
M25	2.48	M155	0.97
M27	1.49	M161	3.39
M28	2.13	M162	1.75
M48	0.00	M207	5.12
M49	1.01	M211	4.62
M50	2.30	M237	0.00

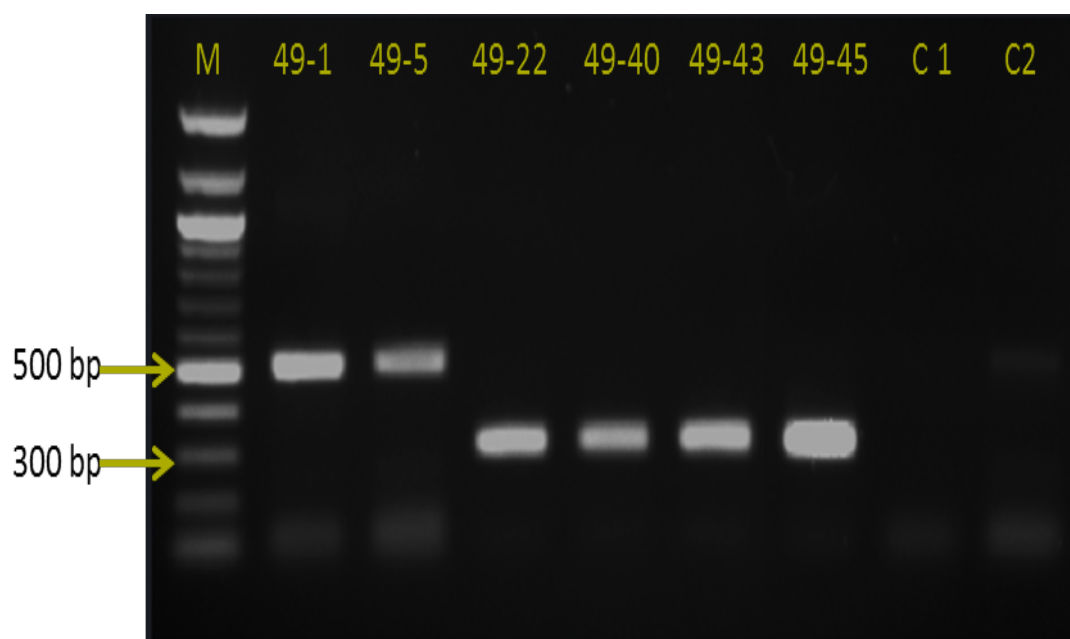
## 6.4.2 Sexual Crossing

### 6.4.2.1 Species Verification

The results of the sequencing verified the species identity of the *Aspergillus terreus* strains (**Appendix 1**) with the exception of strain 49-16 which did not grow within seven days of incubation on the slant. Also, based on ClustalW alignment, the highest match observed for 49-32 was with *A. hortai* standard strain 274 (99.4 %) while the second closest match was with strain *A. terreus* 255 (98.4%) strain of *Aspergillus terreus*.

#### 6.4.2.2 Mating-Type Analysis

Primers ATEM1F and ATEM1R successfully amplified fragments of the *MAT* genes, yielding amplicons of the predicted sizes of 314 bp for *MATI-1* and 513 bp for *MATI-2*. (Figure 6.12).



**Figure 6.12:** Agarose gel (1%) of PCR amplification products of isolates of *Aspergillus terreus* showing mating type amplicons of isolates employed in sexual crosses. The 100bp molecular weight marker is denoted by M; lanes are labelled with the BDUN strain identification code; C1 and C2 are water controls for ATEM1 and ATEM2 respectively.

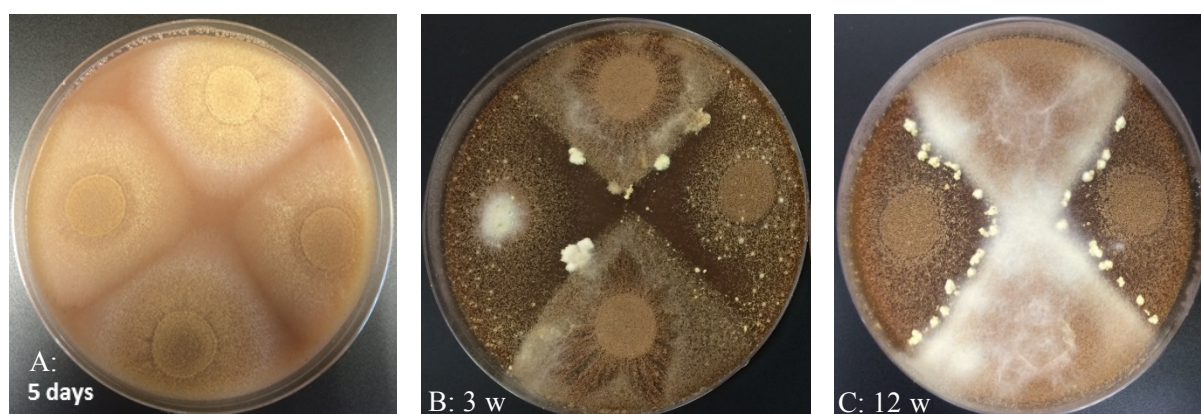
Of the 46 isolates employed in this study, 24 isolates were *MATI-1* and 21 were *MATI-2* as shown, corresponding to 53.3 % and 46.7 % respectively or a near 1:1 ratio (a complete list of mating types are shown in **Appendix 1**). The isolate 49-16 (**Section 6.4.2.1**) was found to yield a bright yellow colony after some weeks and it was investigated for MAT type, however it did not yield any *MAT* product.

#### 6.4.2.3 Induction of Sexual Cycle

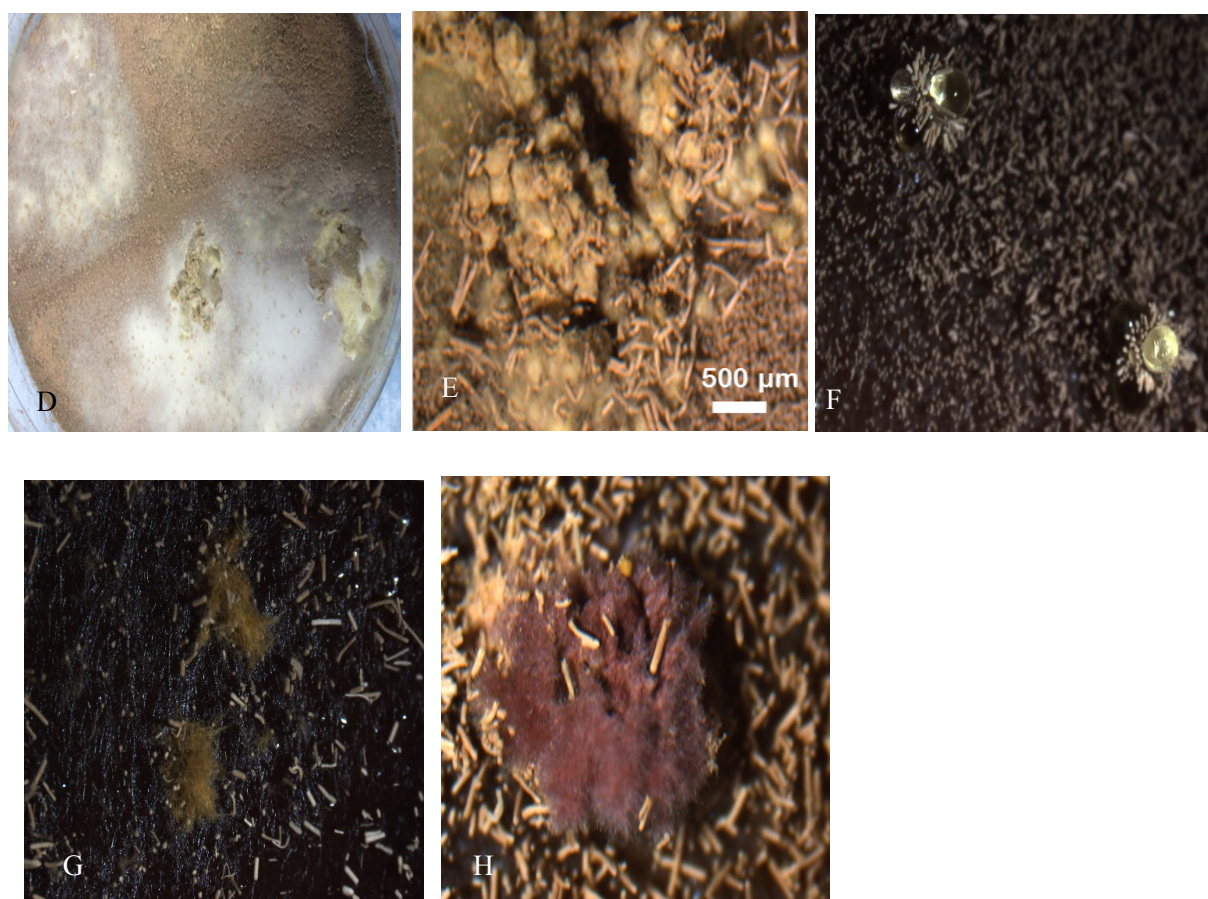
Attempts were made to induce the sexual cycle in compatible *A. terreus* isolates by the barrage crossing method as described by O’Gorman et al. (2009), (Swilaiman *et al.*, 2013),



Bohm *et al.* (2013) and Houbraken and Dyer (2014). Seven representative clinical and environmental strains (four *MAT1-1* strains: 49-22, 49-40, 49-43, 49-45, and three *MAT1-2* strains: 49-1, 49-5, 49-44) were crossed in all possible combinations for a total of 12 weeks. These included crosses with the supposedly sexual fertile isolates 49-40, 49-43 and 49-44 as previously reported by Arabatzis and Velegraki (2012). Plates were incubated upside down to prevent build-up of condensation, a problem which was experienced during initial trials (results not shown). After 5 days, fungal growth had completely covered plates on all growth media (**Figure 6.13 A**). Hyphal aggregation was observed at the barrage zones of the crosses at around two weeks. By the third week, distinct hyphal masses became visible on some crosses (**Figure 6.13 B**). Crosses were incubated for 12 weeks to allow for the possible development of ascospores, and the size and amount of the hyphal masses, where they occurred, was observed to increase during this time (**Figure 6.13 C**).







**Figure 6.13:** Sexual crosses set up between various *Aspergillus terreus* strains of opposite mating types. A: Appearance of a cross on SFA plate after five days; B: Cream-coloured hyphal masses formed at barrage zone on SFA plate; C: Fully mature SFA plate with several masses at barrage zone; D: Crosses of 49-5 on MCA showing formation of white mycelial film, the lower half of the plate has been peeled back to reveal spherical masses; E: Micrograph of the masses showing spherical bodies alongside cylindrical conidiospores aggregations; F: Exudate/liquid droplets found on the surface of 49-5 crosses; G: Hyphal aggregations with a flattened appearance from 49-22 x 49-5 cross; H: Sole pink mass resulting from 49-22 x 49-1 cross.

The hyphal masses were cream to yellow colour and were of varying shapes ranging from spherical to globose and to irregular shapes, ranging in size from ~1 mm – 0.5 cm in diameter. It was observed that within some plates some of the masses grew bigger with prolonged incubation, and others only developed after 3 weeks, though these remained small throughout incubation. Each mature mass contained between 3-28 cleistothecia (**Figure 6.13 C**), and sometimes if they grew close together, two or more masses merged together.

A number of unusual features were observed with crosses of strain 49-5. For instance crosses of 49-5 with 49-43 and 49-45 produced very small hyphal masses both along, and also outside, the barrage zone particularly around the point of inoculation of 49-5. These were

covered in a thin film of white aerial hyphae (**Figure 6.13 D**) and were only visible when the film was removed. In addition some crosses of 49-45 x 49-5 yielded some aggregations that appeared similar to the cleistothecia-bearing hyphal aggregates of other crosses, but were nearly perfectly spherical, cinnamon brown coloured, very numerous and larger than cleistothecia and have similar features to sclerotia (**Figure 6.13 E**). These spherical masses were soft and collapsed upon rolling on tap water agar and were found to be empty, comprising only hyphal tissue. Microscopic examination revealed only structural hyphal material and lots of conidiospores. Also, in crosses of 49-45 with 49-5, slimy droplets of light brown exudate were observed on the surface of the crosses (**Figure 6.13 F**). The 49-22 x 49-5 cross on SFA produced structures which were different from the other hyphal masses observed in that they were flat and of a smaller size than usual (**Figure 6.13 G**).

#### ***6.4.2.4 Effect of Various Parameters on Sexual Reproduction***

The effect of several parameters namely: crossing medium, incubation temperature and effect of air exchange, was investigated on the formation of the hyphal masses in crosses. For these experiments hyphal masses were defined as any cream to yellow, spherical to globose masses from 150-1,000 µm in diameter, consistent with the previous observations of Arabatzis and Velegraki (2013). The total number of sexual structures produced by duplicate plates of the various crosses is presented in **Table 6.10** below. Considerable differences in ability to produce hyphal masses was evident, dependent on isolates used in the crossing work and the environmental conditions as follows.

**Table 6.10:** The effect of three different media, two incubation temperatures and sealing on the total number of sexual reproductive structures produced by isolates of *Aspergillus terreus* after 12 weeks incubation. **Figures** indicate numbers of hyphal masses observed, and are colour coded (red= no hyphal masses; purple = 1-25 masses; dark purple = 25-100 masses; grey= 101-150 masses; green = 151-250 masses).

MEDIUM	OOA				MCA				SFA				TOTAL
TEMP.	32°C		37°C		32°C		37°C		32°C		37°C		
CROSS	Sealed	Open	Sealed	Open	Sealed	Open	Sealed	Open	Sealed	Open	Sealed	Open	
49-22 x 49-1	0	0	0	0	0	0	1	0	0	0	0	1	2
49-40 x 49-1	0	0	0	0	0	0	0	0	0	6	6	6	18
49-43 x 49-1	0	0	0	0	0	0	0	0	1	1	0	0	2
49-45 x 49-1	0	0	0	0	0	0	0	0	0	0	0	0	0
49-22 x 49-5	0	0	0	0	0	0	0	0	0	0	0	0	0
49-40 x 49-5	0	0	7	0	0	0	4	1	0	0	0	0	12
49-43 x 49-5	0	0	13	6	0	0	0	0	15	0	0	0	34
49-45 x 49-5	0	0	2	3	0	0	2	3	0	0	2	0	12
49-22 x 49-44	0	3	1	3	14	0	0	0	113	125	233	119	611
49-40 x 49-44	54	187	8	13	37	2	2	18	27	89	57	164	658
49-43 x 49-44	3	1	0	23	24	24	0	20	75	35	41	43	289
49-45 x 49-44	9	34	11	43	0	0	5	40	47	69	53	58	369
TOTAL	66	238	42	91	75	26	14	82	278	325	392	391	

\*OOA = Odum's Oats Agar; MCA = Mixed Cereal Agar; SFA = Sorghum Flour Agar

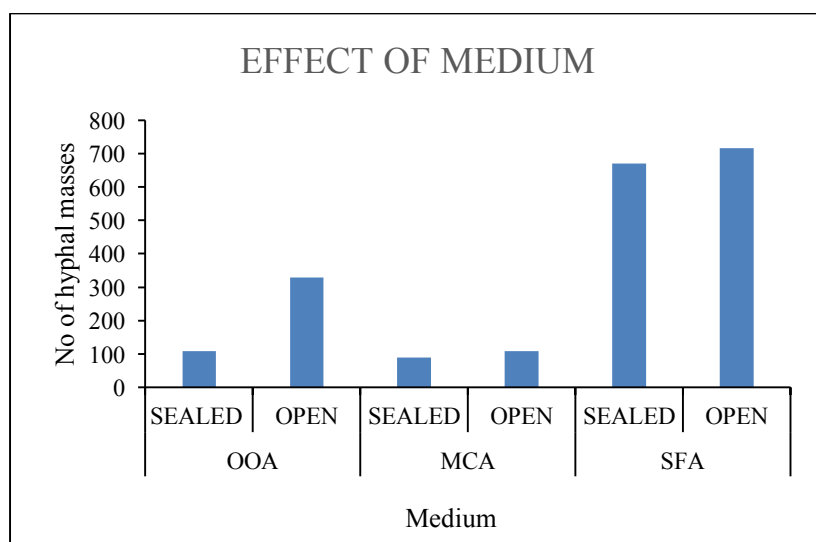
#### 6.4.2.4.1 Variation between Isolates

The *A. terreus* isolates investigated had widely varying capacities to produce sexual structures under the conditions tested. Isolate 49-40 was the most fertile *MAT1-1* isolate across all media while strain 49-44 was the most fertile *MAT1-2* isolate and yielded hyphal masses with all isolates. The most fertile cross was unsurprisingly, 49-40 x 49-44 [a control cross from Arabatzis and Velegraki (2013)] with 658 hyphal masses in total. Isolate 49-1 was the least fertile and did not produce any hyphal masses under several conditions. The cross 49-22 x 49-5 produced small flat yellow aggregation along the barrage zone with no cleistothecia on MCA and SFA while 49-22 x 49-1 resulted in just one pink mass on SFA (not a usual hyphal mass morphology) (**Figure 6.14 G and H**). 49-5 produced unusual, small masses in all crosses (**Figure 6.13 E**) and also very small aggregates which could neither be counted nor isolated for clear observation. Two crosses, 49-45 x 49-1 and 49-22 x 49-5, were unsuccessful across all conditions.

#### 6.4.2.4.2 Effect of Medium Type

Three media namely Mixed Cereal Agar, Odlum's Oatmeal Agar and Sorghum Flour Agar, were compared for efficacy at inducing the sexual cycle in *Aspergillus terreus*. Sorghum flour agar was by far the best medium in inducing sexual structures, producing about 7 times more hyphal masses than MCA and many times more than OOA too. SFA was also consistently better, in both sealed and open plate conditions (**Figure 6.14**).

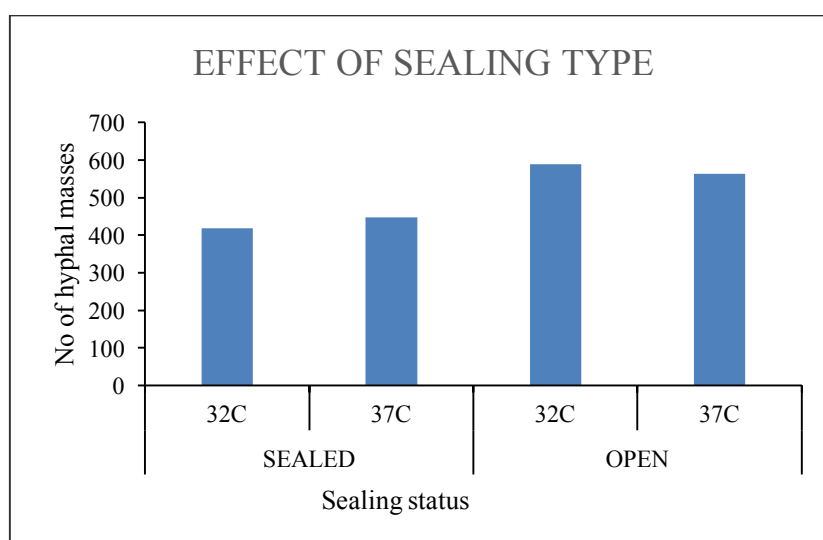
It was observed that in some crosses all media successfully induced sexual reproduction; however in others, such as 49-45 x 49-1 and 49-22 x 49-5 none of the media successfully induced reproduction. In crosses involving 49-1 sexual reproduction was only possible on SFA, with no masses produced on OOA and one empty uncharacteristic mass observed on MCA. Similarly 49-22 only thrived on SFA producing 233, the largest number of sexual structures observed in this work in its cross with 49-44, compared to only one structure that was observed on the other two media. Even the very fertile crosses such as 49-43 x 49-44 had considerably more hyphal masses on SFA than on MCA and OOA (**Table 6.10**).



**Figure 6.14:** Effect of medium type on the formation of hyphal masses during sexual crossing under two conditions when plates were either sealed with Nescofilm, or were placed in an unsealed polythene bag. Media labelling as in **Table 6.10**.

#### 6.4.2.4.3 Effect of Gas Exchange during Incubation

The effect of two types of air exchange during incubation were investigated; namely sealing the plates with Nescofilm (a plastic paraffin film) by wrapping it around individual plates which reduces gas exchange during incubation, as compared with incubating plates (that were not sealed) just inside a plastic bag with the mouth folded under the plates.

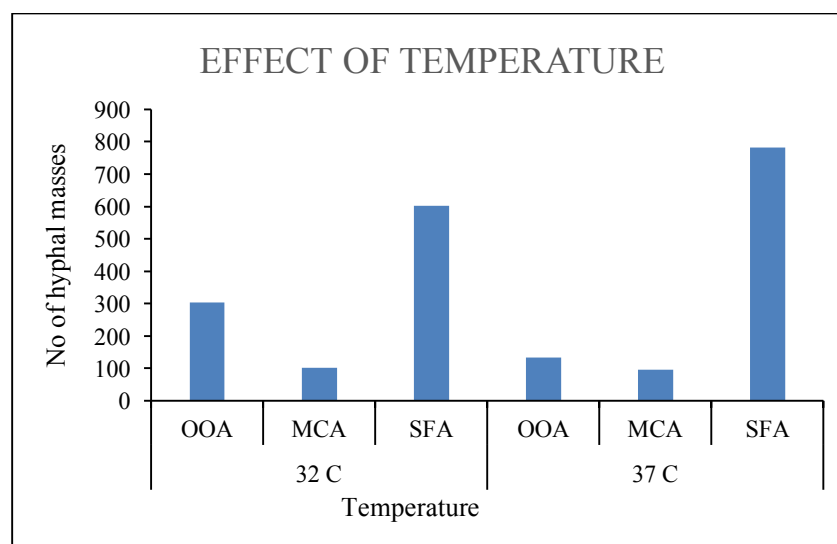


**Figure 6.15:** Effect of air exchange on the formation of hyphal masses during sexual crossing on the three media. Media labelling as in **Table 6.10**.

For all media and regardless of temperature it was found that sealing decreased the amount of sexual structures produced, notably in OOA (**Table 6.10; Figure 6.15**).

#### 6.4.2.4.4 Effect of Temperature

The effect of incubation temperature on the number of sexual reproductive structures produced was investigated. Sexual crossing had previously been reported at 37°C (Arabatzis and Velegraki, 2012). The effect of incubation at a lower temperature, 32°C, was also investigated in this work. It was observed that temperature had no marked effect on the formation of the cleistothecia-bearing hyphal masses. The total number of hyphal masses produced in all crosses at 32°C and 37°C was almost exactly equal with 1,008 and 1,012 masses, respectively (**Table 6.10**). The lower temperature of 32°C was slightly more favourable for hyphal mass production in OOA and MCA while higher temperature of 37 °C was considerably more conducive with SFA medium (**Figure 6.16**). Cross 49-43 x 49-44 was particularly sensitive to temperature, with far more hyphal masses produced at 32°C whereas the higher 37°C temperature favoured the 49-22 x 49-44 cross (**Table 6.10**). However, increased condensation, particularly in sealed plates, was observed at 37°C than at 32°C which made it difficult to observe the plates during incubation. The plates were also observed to dry out more quickly at 37°C than at 32°C, due to the more rapid moisture loss (results not shown).



**Figure 6.16:** Effect of incubation temperature on the formation of hyphal masses during sexual crossing on three media. Media labelling as in **Table 6.10**.

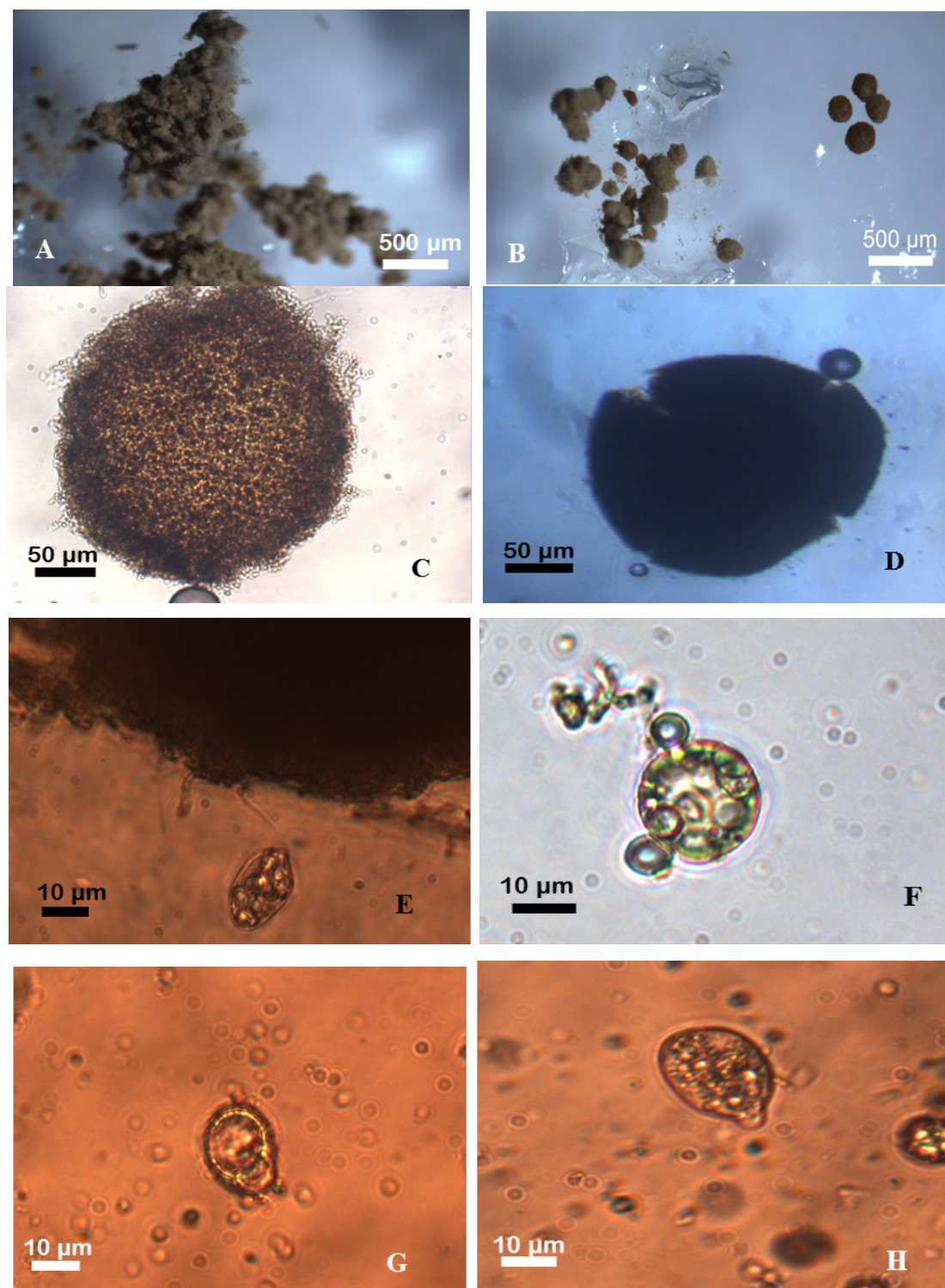
#### 6.4.2.5 Evidence of Genetic Recombination

##### 6.4.2.5.1 Microscopic Examination

Hyphal masses were collected from the crosses at the end of incubation and attempts made to observe sexual structures. Gentle crushing on tap water agar revealed varying numbers of spherical to roughly oval cleistothecia. The mature cleistothecia, ranging in size from 180 - 270  $\mu\text{m}$  (average of 201  $\mu\text{m}$  in diameter based on 10 measurements).

After any remaining hyphal material and conidiospores were removed by rolling along TWA, cleaned putative cleistothecia were gently ruptured and their contents examined for asci and ascospores. Intact asci measuring an average of 13.0  $\mu\text{m}$  were observed from several crosses (**Figure 6.17**), but a large number of asci seen were empty or broken and their contents found spilling out or mixed with the conidiospores (**Figure 6.17**). Most mature asci contained eight spherical ascospores while some contained four or six ascospores. The ascospores were larger than conidiospores measuring an average of 6.4  $\mu\text{m}$  compared to the conidiospores which measure about 2.3  $\mu\text{m}$  in diameter.





**Figure 6.17:** Photomicrographs of sexual structures of *Aspergillus terreus* taken after 12 weeks incubation. A: Hyphal mass under stereomicroscope; B: Mass cleaned of hyphal material on tap water agar to reveal putative cleistothecia; C: Intact mature cleistothecium (49-40 x 49-44 on SFA); D: Ruptured cleistothecium (49-40 x 49-44 on SFA); E: Intact mature ascus with eight ascospores (49-22 x 49-44 on SFA); F: Leaky ascus with six ascospores and within and outside the ascus (49-43 x 49-44 on OOA); G: Developing ascus with few visible ascospores (49-22 x 49-44 on SFA); H: Immature ascus (45 x 44 on OOA).

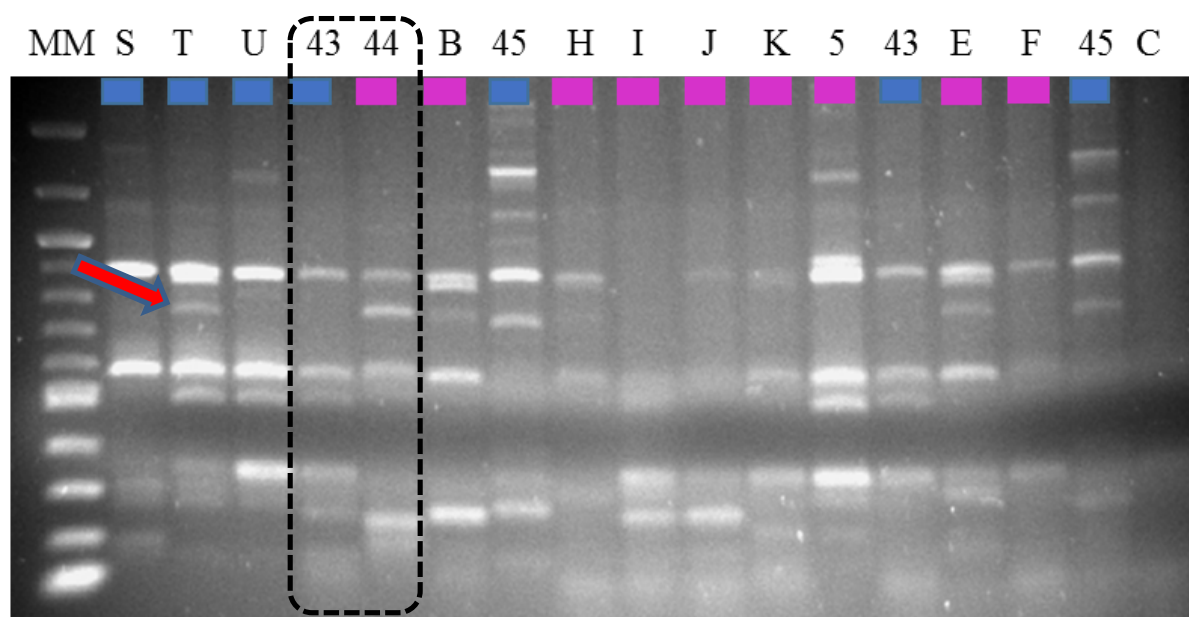


#### 6.4.2.5.2 Single Ascospore Cultures

Upon examination with a stereomicroscope thin, hyaline vegetative hyphae were observed emerging from putative ascospores after overnight incubation and these were subcultured. Residual fragments of the cleistothecia were also observed to be germinating but these were not subcultured. The resultant colonies did not reveal any morphological differences from the parents upon visual and microscopic examination.

#### 6.4.5.2.3 RAPD-PCR of Putative Mature Ascospore Progeny

The use of molecular markers in the characterisation of recombinant progeny is routinely performed to verify the occurrence of sexual reproduction in fungi. The single spore isolates obtained were cultured and DNA obtained from them investigated for meiotic recombination by RAPD-PCR. The fingerprints obtained were compared with parental strains to determine genetic recombination. **Figure 6.19** below shows a representative gel of segregation patterns of the RAPD-PCR markers.



**Figure 6.19:** Evidence of meiotic recombination in progeny from sexual cross between parental strains 49-43 and 49-44 of *Aspergillus terreus* as detected using the AMIII primer. Headings: MM = 100 bp Molecular Marker; Alphabets denote putative ascospore isolates; numbers denote parent strain numbers following the common ID “49-”. C = water control. Blue bars = *MAT 1-1*, pink bars = *MAT 1-2* mating types. Arrow points at diagnostic band in progeny “T” which is a *MAT 1-1* type but exhibits a band from the *MAT-1-2* parent.

Ascospore progeny “T”, obtained using the fourth collection method utilising ethanol, was found to have a similar fingerprint to the *MAT 1-2* parent (49-44) but was of the same *MAT* type as the other parent. This disparity is an indication that genetic recombination had occurred. Several other putative ascospore lines (labelled in alphabetical order in **Figure 6.19**) were investigated for meiotic recombination but no definitive evidence was found except in isolate “T”. This was despite screening with several polymorphic RAPD primers and using the mating type as an additional genetic marker. The recombinant strain “T” was employed in an itaconic acid fermentation to determine the itaconic acid production capacity. The yield obtained was not very impressive at 3.71 g/l which indicates that strain “T” inherited the low itaconic acid producing capacity of parent 49-44 which produced 3.4 g/l in screening experiments (**Figure 4.22**).

### 6.4.3 Fermentation Optimisation

As an alternative to strain improvement, this section now describes attempts to improve itaconic acid yields by the optimising fermentation parameters using statistical Design of Experiments approaches.

#### 6.4.3.1 Half-Factorial Screening of Factors

A completely randomised half-factorial experiment was conducted to screen dependent variables/factors that might significantly influence itaconic acid fermentation based on literature reviewed. The factors varied include temperature, pH, inoculum size and the agitation speed according to the set-up presented in **Table 6.7** for a duration of 4 days. The results are presented in **Table 6.11** and include both the actual IA concentration (g/l) and the yield as a percentage of the theoretical maximum based on glucose consumed.

It was observed that the itaconic acid output varied widely depending on the conditions of the fermentation. In general, itaconic acid concentration and yields (%) were low at low pH values even though the *A. terreus* grew sufficiently as evidenced by mycelial mass values. Similarly, higher temperatures seemed to decrease IA output. The highest yields of 12.04 g/l corresponding to 24.3 % of theoretical maximum IA were recorded in experiment B performed at 33 °C, 180 rpm and pH 3.5 with an inoculum of  $1 \times 10^6$  spores/ml, while the second highest was observed in its duplicate experiment, D. The lowest IA concentration of

0.14 g/l was in experiment P which had a combination of high temperature of 37°C and agitation of 220 and low pH of 2.7.

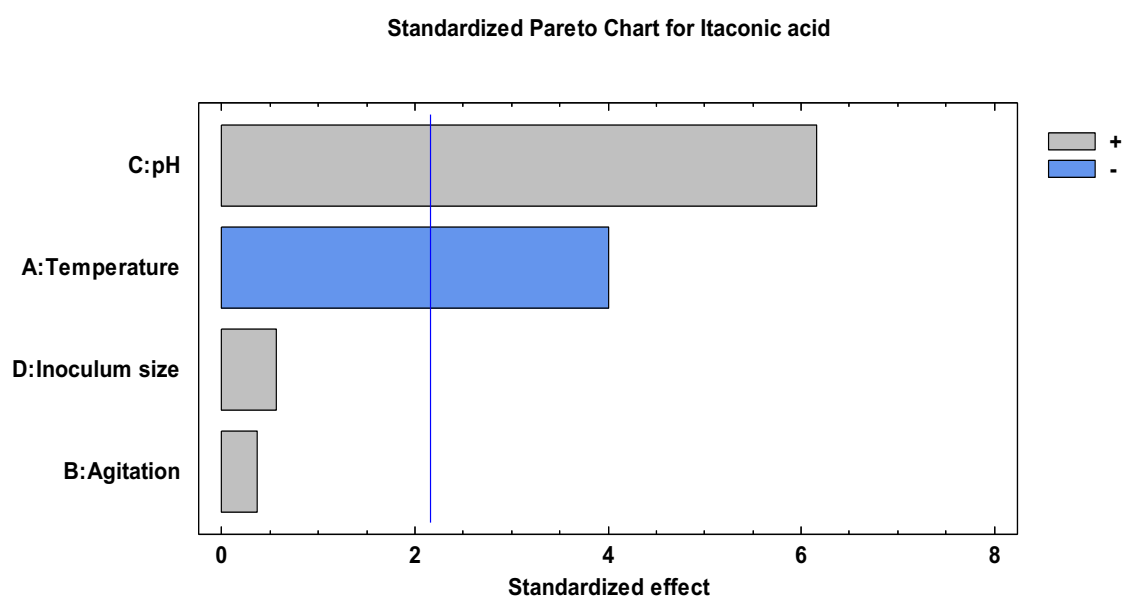
ANOVA for itaconic acid output showed that inoculum size was not statistically significant ( $F = 0.31$ ;  $df = 1$ ;  $P = 0.5842$ ) and neither was agitation ( $F = 0.14$ ;  $df = 1$ ;  $P = 0.7168$ ) whereas pH and temperature were significant factors [ $F = 37.99$ ;  $df = 1$ ;  $P = 0.0000$ ) and ( $F = 16.08$ ;  $df = 1$ ;  $P = 0.0015$  respectively]. The effects of each variable were standardised statistically by dividing the effects of the variable by the standard error, which is equivalent to computing a t-statistic for each effect. The Pareto chart (**Figure 6.20**) illustrates this clearly. The main effects plot (**Figure 6.21**) estimates itaconic acid output as a function of the various parameters investigated. In each plot, the factor of interest is varied from its low level to its high level, while all other factors are held constant at their central values. This plot shows that increasing the temperature results in a decline in the itaconic acid output while increasing pH achieves the opposite. On the other hand, changing agitation rate and inoculum size over the ranges selected did not result in a significant rise.

These details formed the basis for selection of further fermentation parameters, and it was decided that the agitation rate be kept constant while pH and temperature would be varied and inoculum size investigated over a wider range in the response surface method of optimisation.

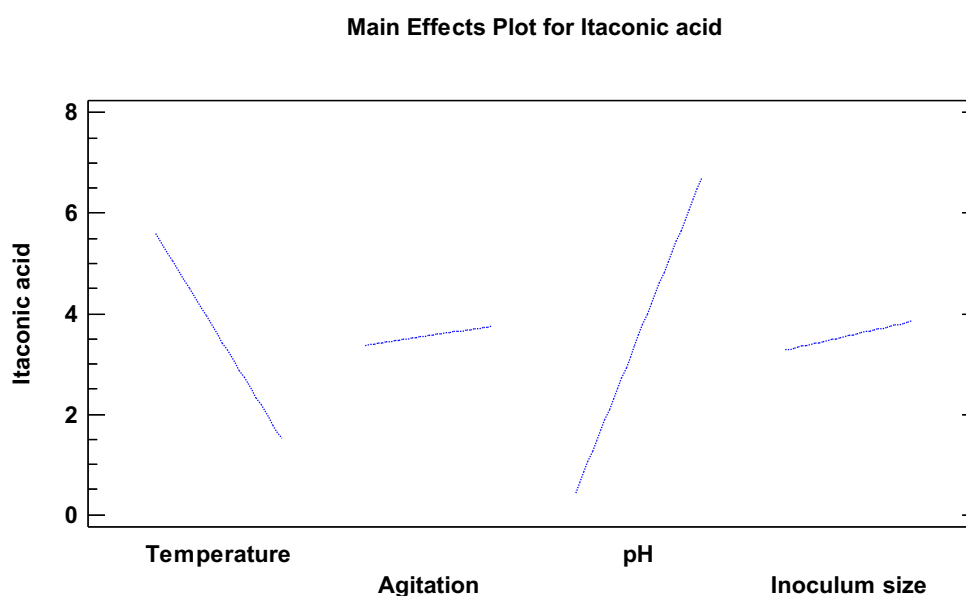
**Table 6.11:** Results of half-factorial design screening of factors important in itaconic acid production by *Aspergillus terreus* 49-22 fermentation on RBDAH.

Block	Experiment Label	Temperature (°C)	Agitation (rpm)	pH	Inoculum size (Log Spores/ml)	Itaconic acid (g/l)	IA Yield (% of TM)*	Mycelial mass (g)
1	I	35	200	3.1	5.813	1.56	5.03	0.57
1	O	37	220	3.5	6	2.49	6.99	0.68
1	A	33	180	2.7	5.48	0.66	3.03	0.37
1	B	33	180	3.5	6	12.04	24.25	1.39
1	K	33	220	2.7	6	0.91	5.45	2.70
1	R	37	220	2.7	5.48	0.16	2.05	0.10
1	E	37	180	3.5	5.48	2.7	7.15	0.67
1	F	37	180	2.7	6	0.14	0.85	0.20
1	L	33	220	3.5	5.48	10.73	21.77	1.07
2	J	35	200	3.1	5.813	2.13	7.35	0.97
2	Q	37	220	3.5	6	4.98	10.03	1.59
2	C	33	180	2.7	5.48	0.28	2.17	0.17
2	D	33	180	3.5	6	9.61	21.36	1.41
2	M	33	220	2.7	6	2.81	14.95	1.13
2	P	37	220	2.7	5.48	0.14	1.36	0.04
2	G	37	180	3.5	5.48	3.26	12.24	0.61
2	H	37	180	2.7	6	0.09	0.78	0.23
2	N	33	220	3.5	5.48	9.58	21.51	1.53

\* This is the percentage of the maximum itaconic acid that can be produced, calculated based on amount of glucose consumed (Section 4.2.2.3).



**Figure 6.20:** Pareto chart showing the variables in decreasing order of importance with the length of each bar being proportional to its standardised effect. Bars extending beyond the vertical blue line correspond to effects which are statistically significant at the 95.0% confidence level.



**Figure 6.21:** Main Effects Plot showing the estimated itaconic acid as a function of the various experimental factors.

#### 6.4.3.2 Factor Optimisation by Response Surface Methodology

The factors selected namely inoculum size, pH and temperature were optimised in a completely randomised, rotatable central composite design in 2 blocks with 2 centrepoints each. Inoculum size was included as a variable because it appeared to improve yields over the narrow range tested; and a third parameter allows for a better RSM design and analyses of data obtained. For instance the estimated response surface mesh plot can only be visualised if there are three independent variables in the design. The behaviour of the factors on itaconic acid yields in fermentation is explained by the second order polynomial equation below.

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{11}X_1^2+b_{12}X_1X_2+b_{13}X_1X_3+b_{22}X_2^2+b_{23}X_2X_3+b_{33}X_3^2 \dots (6.1)$$

Where Y is the predicted response (itaconic acid concentration in g/l),  $b_0$  is a constant,  $b_1$ ,  $b_2$  and  $b_3$  are linear effects,  $b_{11}$ ,  $b_{22}$  and  $b_{33}$  are interaction terms and  $X_1$ ,  $X_2$  and  $X_3$  are the factors temperature, pH and inoculum size respectively.

The results of the fermentation optimisations are shown below (**Table 6.12**). It was evident that itaconic acid was produced in high quantities over only a relatively narrow range of fermentation conditions. The best IA yields obtained were with experiments 9 and 10, with 11.7 g/l and 13.5 g/l produced respectively, which involved fermentation conditions of 30 °C, pH 4.0 and  $1.0 \times 10^6$  spores/ml. Higher temperatures were generally not favourable to yields. For example, experiments 31 and 32 incubated at 43 °C showed no IA production while similar experiment 17 and 18 set up at 35 °C produced up to 4.0 g/l. Low pH also had the same effect, with experiments 13 and 14 performing less well than 15 to 18, and experiments 27 and 28 at pH 4.0 producing about 2.0 g/l IA while 23 and 24 at pH 2.0 produced almost nothing. These trends are best illustrated with the main effects plot (**Figure 6.22**) below.

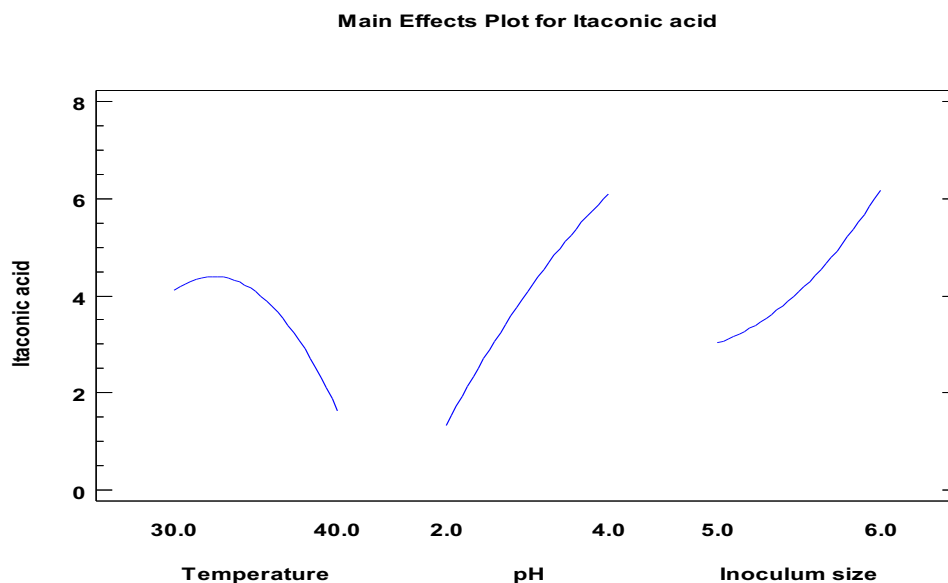
It was however observed that in some experiments while itaconic acid yields (g/l) rose with inoculum size, the yield based on glucose consumed i.e. the % of theoretical maximum declined. This is evident in experiment 1 where just 2.2 g/l IA concentration in the broth corresponds to 50 % of theoretical maximum because of low glucose consumption; while 11.7 g/l concentration in experiment 9 was only 24.3 % of theoretical maximum. Relatively high yields (%) were achieved with the optimisation process resulting in improved IA fermentation efficiencies reaching up to 49.95 % of theoretical maximum possible (**Table 6.12**), with the most efficient condition being temperature of 26.59 °C, pH 3.0 and inoculum of  $\text{Log}_{10}$  5.5.

**Table 6.12:** Central composite experimental design for itaconic acid fermentation and the results obtained.

Block	Label	Temperature (°C)	pH	Inoculum (Log <sub>10</sub> )*	Itaconic acid (g/l)	IA Yield (% of TM)†	Mycelial Mass (g)
1	15	35	3.0	5.5	4.01	34.77	0.49
1	21	35	3.0	6.341	10.89	26.16	0.77
1	7	30	4.0	5	4.77	25.50	0.56
1	19	35	4.68	5.5	6.50	19.99	0.50
1	29	40	4.0	6	2.04	5.46	0.67
1	1	26.59	3.0	5.5	2.22	49.95	0.16
1	31	43.41	3.0	5.5	0.00	0.00	0.25
1	3	30	2.0	5	0.13	3.01	0.08
1	25	40	2.0	6	0.00	0.00	0.00
1	16	35	3.0	5.5	3.94	28.15	0.26
1	27	40	4.0	5	2.01	15.47	0.68
1	11	35	3.0	4.659	1.58	31.51	0.28
1	5	30	2.0	6	0.26	27.99	0.00
1	9	30	4.0	6	11.69	24.33	0.60
1	13	35	1.32	5.5	0.00	0.00	0.00
1	23	40	2.0	5	0.13	9.99	0.00
2	17	35	3.0	5.5	4.09	34.08	0.38
2	22	35	3.0	6.341	10.17	28.03	1.10
2	8	30	4.0	5	6.51	22.37	0.49
2	20	35	4.68	5.5	7.40	23.48	0.48
2	30	40	4.0	6	2.32	6.64	0.74
2	2	26.59	3.0	5.5	2.14	38.98	0.14
2	32	43.41	3.0	5.5	0.00	0.00	0.21
2	4	30	2.0	5	0.00	0.00	0.12
2	26	40	2.0	6	0.00	0.00	0.00
2	18	35	3.0	5.5	3.99	20.80	0.59
2	28	40	4.0	5	1.95	10.89	0.66
2	12	35	3.0	4.659	1.29	31.04	0.34
2	6	30	2.0	6	0.26	25.81	0.06
2	10	30	4.0	6	13.54	28.32	0.65
2	14	35	1.32	5.5	0.00	0.00	0.00
2	24	40	2.0	5	0.00	0.00	0.00

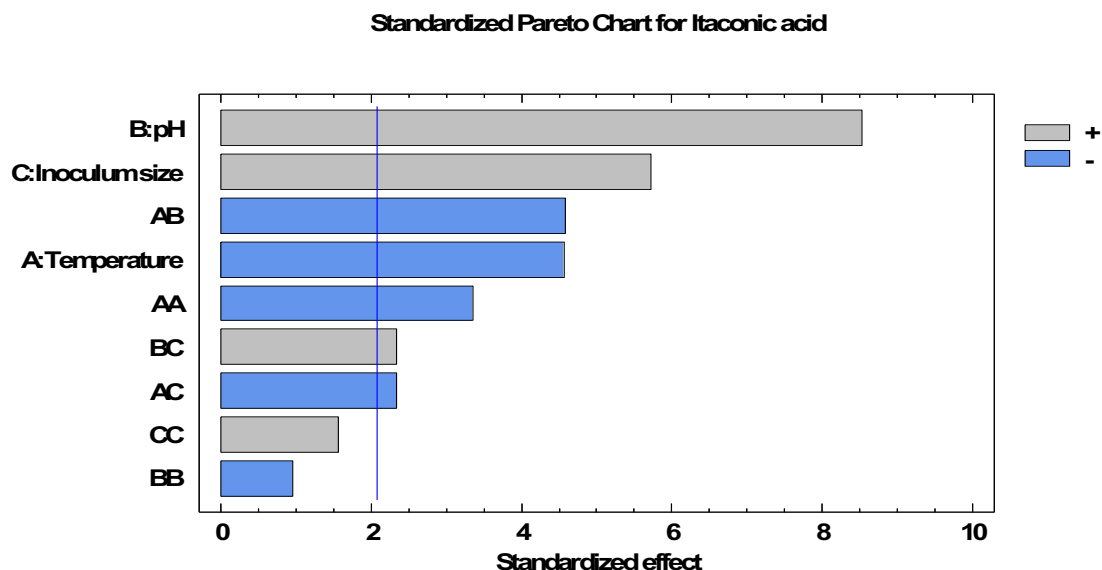
\* Log<sub>10</sub> 4.659, 5, 5.5, 6 and 6.341 represent final inoculum concentrations of  $4.56 \times 10^4$ ,  $1 \times 10^5$ ,  $3.16 \times 10^5$ ,  $1.0 \times 10^6$  and  $2.19 \times 10^6$  spores/mL respectively.

† This is the percentage of the maximum itaconic acid that can be produced, calculated based on amount of glucose consumed (**Section 4.2.2.3**).



**Figure 6.22:** Main Effects Plot showing the estimated itaconic acid as a function of the various experimental factors.

The Pareto chart for itaconic acid production (**Figure 6.23**) shows that while all factors were significant, the most important variable in the optimisation process was pH, followed by inoculum size and then temperature. The interactions between all three variables were also significant at the 95 % confidence level (**Figure 6.23**).



**Figure 6.23:** Pareto chart showing the estimated effects in decreasing order of importance. Values extending beyond the vertical blue line are statistically significant at the 95.0% confidence level.



A response surface optimisation analysis of the data to investigate the relationship between these variables yielded a prediction model for itaconic acid production, and it is shown in equation 6.2 below (where  $X_1$  = Temperature,  $X_2$  = pH and  $X_3$  = Inoculum size).

$$\text{Itaconic acid concentration, } Y = -81.5761 + 6.01X_1 + 6.92X_2 - 13.87X_3 - 0.05 X_1^2 - 0.35 X_1X_2 - 0.35 X_1X_3 - 0.34X_2^2 + 1.76 X_2X_3 + 2.20X_3^2 \dots\dots\dots (6.2).$$

The experimental data obtained from the RSM optimisation fitted the prediction model with a  $R^2$  of 89.13 % (**Table 6.13**) and this model was used to provide estimated results of the various experiments performed. The results are presented in **Appendix 7**, together with their 95 % confidence limits.

**Table 6.13:** Analysis of variance for optimisation of itaconic acid production on RBDAAH by RSM

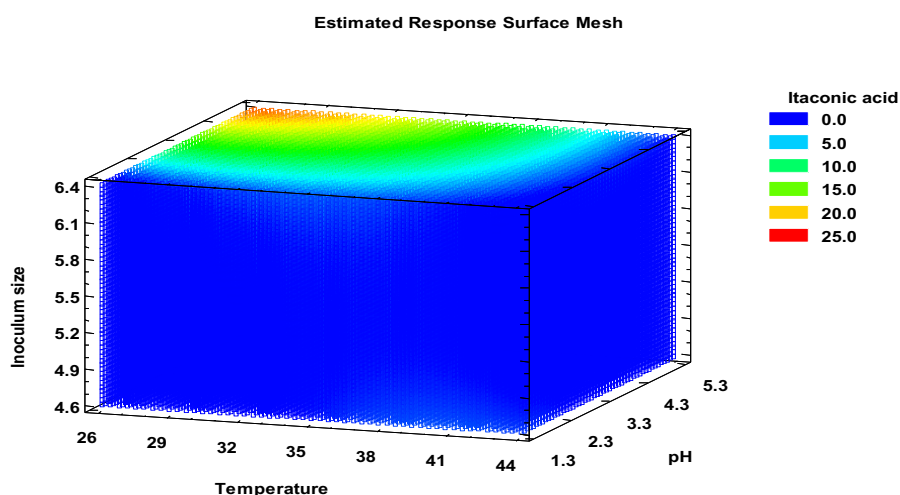
Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
$X_1$ :Temperature	47.5922	1	47.5922	20.82	0.0002
$X_2$ :pH	166.401	1	166.401	72.79	0.0000
$X_3$ :Inoculum size	74.8274	1	74.8274	32.73	0.0000
$X_1^2$	25.7852	1	25.7852	11.28	0.0028
$X_1X_2$	47.942	1	47.942	20.97	0.0001
$X_1X_3$	12.3836	1	12.3836	5.42	0.0295
$X_2^2$	2.09678	1	2.09678	0.92	0.3486
$X_2X_3$	12.4018	1	12.4018	5.42	0.0294
$X_3^2$	5.61293	1	5.61293	2.46	0.1314
Total error	50.2936	22	2.28607		
Total (corr.)	462.87	31			

Note: R-squared = 89.1344 %; R-squared (adjusted for d.f.) = 84.6894 %;

Standard Error of Est. = 1.51198; Mean absolute error = 1.09147

Durbin-Watson statistic = 1.81925 (P=0.3268); Lag 1 residual autocorrelation = 0.0771723

In order to explore the effect of fermentation parameters on itaconic acid production, a response surface mesh plot was created by plotting the estimated itaconic acid yields in g/l (response) as a function of the independent variables. The three axes represents the variables while the predicted values are represented by points in various colours as explained in the key. The estimates are shown in **Figure 6.24** below.



**Figure 6.24:** Response surface mesh showing effect of the interactions of the various parameters on predicted IA yields.

#### 6.4.3.3 Optimisation Verification and Scale Up

The optimal conditions for maximum itaconic acid production when using RBDAAH in fermentations were predicted by the Statgraphics software and are presented in **Table 6.14**. It was predicted to attain a possible optimum value of **19.64 g/l**. These conditions differ from those observed to produce the highest yields from **Table 6.15** of 30 °C, pH 4.0 and Log<sub>10</sub> 6.0 spores/ml inoculum. Consequently, it was decided that both sets of conditions would be investigated. The findings are presented below (**Table 6.15**).

**Table 6.14:** Conditions for response optimisation of itaconic acid fermentation.

FACTOR	LEVEL		
	LOW	HIGH	OPTIMUM
Temperature (°C)	26.59	43.41	26.59
pH	1.32	4.68	4.68
Inoculum size (Log <sub>10</sub> )	4.66	6.34	6.28

**Table 6.15:** Itaconic acid yields from the optimum conditions selected from the CCD experiments and the optimum conditions predicted by the model for fermentation in RBDAAH

CONDITION	TEMP. (°C)	pH	INOCULUM (Log <sub>10</sub> )	ITACONIC ACID (g/l)	ITACONIC ACID YIELD (%)
SELECTED OPTIMUM CONDITIONS	30.00	4.00	6.00	11.96 ± 0.53 <sup>a</sup>	41.07 ± 2.64 <sup>a</sup>
PREDICTED OPTIMUM CONDITIONS	26.59	4.68	6.28	7.90 ± 0.23 <sup>b</sup>	36.75 ± 2.66 <sup>b</sup>

<sup>a,b</sup> Values within columns bearing different alphabet superscripts are significantly different

It was observed that there was a significant difference between the yields obtained from the optimum conditions selected visually from **Table 6.12** (for experiments 9 and 10) and conditions that the model predicted ( $F = 67.59$ ;  $df = 1$ ;  $P = 0.0012$ ). The predicted conditions from the modelling surprisingly resulted in both lower itaconic acid concentrations and yield (%) based on theoretical maximum than the previously selected optimal conditions. The conditions selected from **Table 6.15** were therefore utilised in two repeat experiments to ensure reproducibility, with average yields of 11.87 and 13.66 g/l IA obtained which were found to be consistent (data not shown). The same conditions were therefore employed in final subsequent experiments where the fermentation was scaled-up from 25 ml to 200 ml and 500 ml volumes in 2L and 5 L flasks respectively, and the results are presented in **Table 6.16**.

It was evident that both the actual itaconic acid amounts (g/l) and yields (%) considerably improved in the scale-up experiments, with the 200 ml fermentation producing 13.9 g/l with a conversion efficiency of almost 50%. Similarly, the 500 ml fermentation achieved a 16.26 g/l yield which was 46.1 % of the theoretical maximum possible.

**Table 6.16:** Scale-up fermentation experiments for itaconic acid production from RBDAH. Values are averages of triplicates, with the standard deviations.

<b>Fermentation Volume</b>	<b>Itaconic acid yield (g/l)</b>	<b>Itaconic acid yield (%) of Theoretical maximum</b>
200 ml	13.94 ± 1.34	49.63 ± 4.76
500 ml	16.26 ± 0.37	46.09 ± 1.04

The amounts of IA produced in the scale up experiments were found to be significantly different ( $F = 8.43$ ;  $df = 1$ ;  $P = 0.0440$ ) while the glucose utilisation efficiencies, i.e. the yield as a function of theoretical maximum did not change significantly between the 200 mL and 500 mL fermentations ( $F = 1.59$ ;  $df = 1$ ;  $P = 0.2759$ ).

## 6.5 Discussion

Three methods were employed to attempt to improve the yield of itaconic acid fermentation by two isolates of *A. terreus* 49-5 and 49-22, namely ultraviolet mutagenesis, sexual crossing and optimisation of the fermentation process itself. These gave mixed results as follows.

### 6.5.1 Ultraviolet Mutagenesis

The UV mutagenesis was performed on both strains and in a rationalised screening process employing the use of gradient concentration method performed.

#### 6.5.1.1 Itaconic Acid Tolerance

Firstly, an itaconic acid tolerance test was performed to determine the range of itaconic acid to include in a rationale screening medium for potential high-yielders. The logic for this approach was that IA production can be inhibitory to the growth of *A. terreus*. Therefore mutants with higher tolerance to IA should correspondingly be able to tolerate higher levels of production of IA (Yahiro et al. 1995). It was observed that the test isolates could only tolerate about 4.0 g/l of itaconic acid in solid media with no growth observed at higher

concentrations. This contrasted with the higher amounts of IA that they were able to withstand in submerged fermentations according to work elsewhere in the present study. This difference may be because the IA was present in the solid medium at concentrations inhibitory to the germination process, whereas in the fermentation process IA is produced after the organism has started growing and will have attained tangible mycelial mass formation before end-product toxicity sets in. Consequently, the gradient plates were prepared in a way to ensure that the maximum 30 g/l IA concentration was at the extreme right end such that the supposed maximum tolerance limit of 4.0 g/l was included in the gradient plate to ensure survival and germination of some mutant spores. This compares to the higher maximum value of 60 g/l used by Yahiro et al. (1995) in the media for their screening work.

#### **6.5.1.2 Kill Curve**

Classical kill curves were then obtained, which allowed prediction of 5% survival levels. This 5% level was chosen as a standard point being used for UV mutant selection although it is acknowledged that different survival levels will lead to different mutational characteristics (Simpson and Caten, 1979; Han and Parekh, 2005; Parekh, 2009; Dyer *et al.*, 2017). It is noted that the kill curves may indicate the presence of non-genotypic heterogeneity both in and among isolates of *Aspergillus terreus*. This is a trait whereby individual cells within clonally-derived populations, with the same genetic background, display differences when microbial cells are exposed to harmful stressors (Hewitt *et al.*, 2016) such as mutagens.

#### **6.5.1.3 Screening and Fermentation**

A total of 250 UV mutants were selected which appeared to show increased tolerance to IA. Of these, 90 mutants which showed the highest IA tolerance on IAPDA plates were selected for further analysis. The fermentation with these mutants yielded very promising results with several strains matching or surpassing the performances of the parental strains after fermentation for seven days in 12-well boxes (**Figure 6.9**). A total of seven mutants were recovered which produced higher than the parental 49-22 strain, up to a 3.5-fold increase observed in itaconic acid yields. This was an interesting observation as it has been observed that in yield improvement during antibiotic production for example, mutations do not usually result in over 10 % increase in product titres, and thus the improvements are more likely to be small increments in output than drastic increases (Hunter, 2007; Parekh, 2009). The fact that

only eight positive variants were recorded out of a total 250 mutants contrasted with the findings of Simpson and Caten, (1979) who observed a tendency of population mean yields to decrease following mutagenesis, but with the same ratio of positive and negative variants. An analysis of the trend of the mutant fermentations revealed that yields were normally distributed in mutants of both parental strains, which is similar to observations in antibiotic production by mutants as reported by Hunter (2007). The low number of high-titre mutants observed were also in line with reports that few mutants perform better than the parents after random mutagenesis after allowing for experimental error (Hunter, 2007). However, a repeat fermentation with these supposed high-yielding strains indicated that they were all relatively low-producers, indeed below the original parental isolates. This situation could be due to a reversion of the mutants to the wild type which is a common occurrence with point mutations such as those induced by UV mutations (Maheshwari, 1999). Also, even though this was disappointing, it is not unusual to observe such false positives in mutagenesis screens and it has been reported that most high-yielding mutant isolates turn out not to have improved titres (Hunter, 2007).

The fact that high-titre mutants were eventually not observed could be due to a number of reasons. Firstly, the use of UV light causes random mutations in the organism's genome and may not cause the specific changes in the pathway required to bring about an increase in IA yields such as an increase in expression of *cis*-aconitate decarboxylase gene (*CADI*) in mutants. Secondly, the UV mutagenesis rate may not have been particularly efficient at inducing the desired non-lethal mutations compared to other methods available that were not tested due to time limitations. Alternative methods of mutagenesis include chemical methods, and it has been reported that near-UV light mutation in the presence of 8-methoxypsoralen was the least effective method in inducing variation in penicillin titres in *Aspergillus nidulans* (Simpson and Caten, 1979). Thirdly, this work employed mutagenesis with 5 % survival rate but either higher or lower survival rates might have been more effective at producing higher-titre mutants. For example, Simpson and Caten (1979) obtained best yields from mutagenic treatments with 0 – 4 % survival. Next, it is possible that the UV mutants exhibited reversion back to the parental phenotype. This can sometimes be overcome by incubating mutants in the dark after mutagenesis to prevent photo-activated repair. In addition, the gradient plate technique of IA Yahiro *et al.* (1995) may not have been reliable as during incubation diffusion of solute through the agar will have occurred to some extent. Finally, it might have been simply that insufficient numbers of UV mutants have been screened. A total

of 250 mutants were examined in the present study, whereas several hundred more and even thousands of mutants might need to be screened to obtain the desired phenotype. This was not possible in the present study due to time constraints associated with the lengthy screening and fermentation processes.

Very limited information exists on the successful application of mutation for the improvement of itaconic acid yields in literature, with the one successful report being the TN-484 mutant obtained from IA screening of 670 colonies obtained after *N*-methyl *N'*-nitro-*N*-nitrosoguanidine treatment (Yahiro *et al.*, 1995). This mutant was able to produce 65 g/l IA and a yield of 0.54 g/g based on glucose consumed which was 1.2 times that of the parental strain. This 1.2-fold increase is attributable to the fact that in a later report, it was observed that the mutant TN-484-M1 [reported to be the same strain by Dwiarty *et al.*, (2002)] demonstrated a 5-fold transcription level of *CAD1* than the native strain (Kanamasa *et al.*, 2008). The low frequency of reports involving high-titre mutants, the need to screen a large number of colonies even after prescreening in a rationalised selection process to obtain a high-yielding mutant (Yahiro *et al.*, 1995), and the absence of stable high-yielding mutants in this work indicate that itaconic acid yield increase by mutation might not be a straightforward task.

### **6.5.2 Sexual Crossing**

The use of sexual reproduction to induce genetic recombination in heterothallic ascomycetes has been well-reported (Böhm *et al.*, 2013; Swilaiman, 2013; Ropars *et al.*, 2014). *Aspergillus terreus* was previously thought to be asexual and lack a sexual cycle. However the sexual stage was recently discovered by Arabatzis and Velegraki (2012), indicating that this industrially important species is now a candidate for strain improvement by sexual crossing (Ashton and Dyer, 2016). There have so far been no reports of the use of sexual recombination to improve yields of valuable extrolites such as itaconic acid and lovastatin in *A. terreus*. Therefore, attempts were made to induce a sexual stage between high-yielding *A. terreus* strains of opposite mating type in an attempt to generate progeny with fresh genetic diversity, which might hopefully include some with improved yields of itaconic acid.

#### **6.5.2.1 Mating-Type Analysis**

The *Aspergillus terreus* isolates in the BDUN collection were verified by amplifying regions of the genes coding beta-tubulin and calmodulin. The sequences obtained were then

compared with the BLAST database and all strains were found to indeed be *A. terreus* isolates except for 49-16 which yielded no product with the primers used. This step was important as strains which have been classed as *A. terreus* using classic identification methods have recently been reclassified as different species (Balajee *et al.*, 2009).

Investigations using a PCR-based mating-type diagnostic test revealed an approximately 1:1 ratio of *MAT1-1* and *MAT1-2* mating types when 38 *A. terreus* isolates were sampled. This is similar to the findings of Paoletti *et al.* (2005), O’Gorman *et al.* (2009) and Swilaiman *et al.* (2013) concerning other heterothallic *Aspergillus* species, and is consistent with the predicted ratio for fungal species with a sexual cycle (Dyer and Paoletti, 2005).

#### **6.5.2.2 Induction of a Sexual Cycle**

Attempts were made to induce the sexual cycle of *A. terreus* by using the barrage method of sexual crossing between isolates of opposite mating type under conditions recently reported to have successfully induced sexual reproduction in *A. terreus* (Arabatzis and Velegraki, 2012). Various other conditions, such as use of OOA medium which has been successfully used to induce sexual reproduction in many other aspergilli (Dyer and O’Gorman, 2012), were also used. This resulted in the formation of hyphal masses which were first observed after two weeks, with the numbers and sizes increasing slightly over time. This indicated the possibility that sexual reproduction was occurring. The number of hyphal masses produced varied according to growth conditions and particular isolate strain used for crossing.

##### **6.5.2.2.1 Macroscopic and Microscopic Features**

Hyphal masses were observed to be cream coloured and irregularly shaped. They were formed in an “X” shape at the barrage zone and were similar to those reported by Arabatzis and Velegraki (2012). They were observed in many cases outside of the barrage zone in some plates, particularly around the inoculation points. These masses were found to be composed of clusters of cleistothecia, which were wrapped within hyphal material to varying extents. They were easily separated on tap water agar under a stereomicroscope to reveal up to 28 cleistothecia, particularly where Sorghum Flour Agar (SFA) and Mixed Cereal Agar (MCA) were used as the growth media. This compares to the maximum of five cleistothecia reported by Arabatzis and Velegraki (2012) to be present in such hyphal masses. This feature



(formation of cleistothecia in clusters) makes the teleomorph of *A. terreus* similar to that of *Fennellia flavipes* (Arabatzis and Velegraki, 2012).

Other hyphal structures were also observed in some crosses. However, these were found to be barren (i.e. lacking ascospores), and were soft and easily crushed. These were mainly formed in crosses with isolate 49-5 (**Figure 6.14 E**). These structures appeared to be sclerotia-like structures similar to the description of “pseudosclerotia” made by Samson *et al.* (2011) in their report of *A. neoaffricanus*, a new species formerly referred to as *A. terreus* var. *affricanus*. Similar structures were observed by Arabatzis and Velegraki (2012).

### **6.5.2.3 Effect of Various Parameters on Sexual Reproduction**

#### **6.5.2.3.1 Variation between Isolates**

There was considerable variation between isolates in their ability to produce hyphal masses in crosses. The most prolific strains were 49-40 (*MAT1-1*) and 49-44 (*MAT1-2*), which were obtained from the original study of Arabatzis and Velegraki (2012), who also found these to be the most sexually fertile isolates in their screenings when using MCA as the growth medium (Arabatzis and Velegraki, 2012). These “supermaters” produced the highest number of hyphal masses in the cross 49-40 x 49-44 across all conditions (in total 658 masses were formed), compared to cross 49-22 x 49-44 which had the second highest number (601 masses formed in total). All crosses involving 49-44 performed well regarding the production of hyphal masses. However, many other isolates exhibited much lower fertility, indeed failing to form hyphal masses under most conditions assayed. This is similar to the findings of Arabatzis and Velegraki (2012), who also found that many *A. terreus* isolates failed to form cleistothecia in crosses, and variations in sexual fertility have been reported for many fungal species (Houbraken and Dyer, 2015). The least fertile strain was 49-1 with 2, 18, 2, and 0 masses with *MAT1-1* strains 49-22, 49-40, 49-43 and 49-45 respectively (**Table 6.10**). Similarly 49-5 was poor in hyphal mass formation, producing 0, 12, 34 and 12 masses with the same *MAT1-1* strains. It is observed that the recently isolated 49-43 and 49-40 (Arabatzis and Velegraki, 2012) performed best with 49-5 and 49-1 respectively producing 34 and 18 masses respectively; but when 49-22 (*MAT1-1*) is crossed with these two strains, only two masses were observed with 49-5 and no mass with 49-1. Considering that their *MAT* genotype s were repeatedly determined to rule out mis-matching of mating types (**Figure**

6.12), it might be that these strains may have lost most of their ability to reproduce sexually with time. It has been reported that isolates maintained for long periods of time in axenic culture are prone to a ‘slow decline’ in fertility compared to freshly isolated strains (Dyer and Paoletti, 2005), which might in part explain the variation in fertility in *A. terreus* observed in the present study. Since this strain has been in the collection for longer than, for example, 49-40 through to 49-44, this may partly explain the disparity in performances between the earlier strains and the latterly obtained strains.

#### 6.5.2.3.2 Effect of Medium Type

The number of hyphal masses produced on OOA and MCA were fairly similar (**Figure 6.9**). By contrast, SFA performed consistently better than both media across all crosses. Indeed, SFA was the only medium on which some crosses (e.g. 49-43 x 49-1 and 49-40 x 49-1) were successful (**Table 6.10**). This was a very significant result as it suggests that sorghum flour agar might be a particularly favourable medium upon which to induce sexual reproduction in *Aspergillus* species, and perhaps more broadly in the pezizomycotina, given that previous screening work had shown that OOA and MCA were the best media for inducing sexual cycles in ‘fastidious’ *Aspergillus* and *Penicillium* species (Dyer and O’Gorman, 2011; Arabatzis and Velegraki, 2012). It appears as if the SFA medium is better when left unsealed as the counts on this medium were higher in the plates incubated in the polythene bags. The counts were similar in MCA and OOA with the exception of the supermater pair of 49-40 x 49-44 at 32 °C which contained 187 masses on OOA. However, curiously this pair performed poorly on MCA at both temperatures compared to OOA which is contrary to the reports of Arabatzis and Velegraki (2012). The reason for this is not clear, but might relate to the use of different batches of MCA, as this is derived from a natural source with inherent variation in composition.

#### 6.5.2.3.3 Effect of Gas Exchange During Incubation

The degree of gas exchange had a significant impact on formation of hyphal masses in *A. terreus*. Crossing plates were either sealed with paraffin tape (high restriction of gas exchange) or 3 plates were stored together in a folded polythene bag (low restriction) during incubation. Overall, sealing was found to result in a considerable decrease in hyphal mass formation. This contrasts with reports that sealing increases number of cleistothecia formed

in *A. nidulans* (Zonneveld, 1977) but is in agreement with findings that in *Eurotium* species restricted gas exchange impedes cleistothecia formation (Ashour, 2014). Sealing increases CO<sub>2</sub> accumulation and lowers oxygen availability, and these factors presumably influence sexual reproduction. However, in *A. terreus* the effect was partly dependent on the medium and temperature used. For example, when using OOA, sealing decreased the number of hyphal masses at both 32 °C and 37 °C, whereas with MCA, sealing enhanced sex at 32 °C while impeding it at 37 °C. With SFA, sealing appeared to have no impact at 37 °C, while it impeded sex at 32 °C (**Table 6.10**). Storing plates in the folded polythene bags also had the advantage of decreasing the rates of plates drying out compared to plates incubated without plastic bags while also decreasing condensation compared to sealed plates (results not shown). It is also worth noting that while the effect of stacking on crossing outcomes was not investigated it was observed that the bottom plate in the stack of 2 had a higher residual volume of agar at the end of the incubation while in some cases, the top plate was showing signs of drying out after 12 weeks. This was more obvious in the bagged plates and less so in the sealed plates. In addition, previous work has shown that the number of plates stacked up together can influence sexual fertility (Ashour, 2014; AA Ashour and PS Dyer, unpublished results), so this factor warrants further attention in future studies.

#### 6.5.2.3.4 Effect of Temperature

Temperature effected the number of hyphal masses formed depending on the medium used, with temperature well known to influence sexual fertility in many other *Aspergillus* species (Dyer and O’Gorman, 2012). In the case of *A. terreus*, the effect of temperature was dependent on the medium type. When using SFA incubation at 37 °C improved hyphal mass formation considerably, whereas the higher temperature was less favourable for OOA whilst for MCA there was no clear impact of temperature. Overall, the number of hyphal masses produced at both temperatures were almost exactly the same, with 1008 structures at 32 °C and 1012 structures at 37 °C (**Table 6.10**).

#### 6.5.2.4 Evidence of Genetic Recombination

#### 6.5.2.4.1 Microscopic Examination

The masses from various crosses were examined under a compound microscope for evidence of ascus and ascospore formation. The putative cleistothecia were found to be composed of hyphae thinner than the normal *A. terreus* hyphae, wrapped in a tight spherical mass similar to the report of Arabatzis and Velegraki (2012). Cleistothecia were on average 201 µm in diameter which is larger than the size reported by Arabatzis and Velegraki (2012). The cleistothecia were easily crushed, with the exudate comprising hyphal material, putative ascospores and a few intact asci. The intact asci were observed to contain between 4, 6 or 8 ascospores, which is similar to the report of Arabatzis and Velegraki (2012) that the asci contained 6 or 8 spores. Putative ascospores were spherical, transparent and colourless, measuring on average 6.4 µm in length and were thus considerably larger than conidiospores. These characteristics of the teleomorph of *A. terreus*, namely possession of smooth-walled, hyaline to pale yellow ascospores with an inconspicuous equatorial groove or ridge, make it similar to *Fennellia flavipes* (Arabatzis and Velegraki, 2012). It was thus verified microscopically that these observed structures of *Aspergillus terreus* were indeed sexual structures cleistothecia, asci and ascospores.

#### 6.5.2.4.2 Pure Ascospore Cultures and Rapd-PCR Verification of Recombination

There were several challenges to obtain clonal ascospore cultures. This was in part due to the fact that the cleistothecia were surrounded by large amounts of conidiospores from the parental strains in the cross. Various methods were employed to try and obtain pure ascospores, including rolling cleistothecia on tap water agar to remove adhering conidiospores before crushing the cleistothecia to obtain pure ascospore cultures, as routinely used for other *Aspergillus* and *Penicillium* species (e.g. Bohm *et al.*, 2013; Swilaiman *et al.*, 2013). RAPD-PCR banding patterns, in combination with the mating-type, were then used as genetic markers to screen for recombinant offspring. Despite this, almost all colonies obtained were of the same genotype of one of the two parental strains i.e. efforts failed to identify novel recombinant lineages. However, a single apparent recombinant progeny labelled “T” was obtained from a cross between isolates 49-43 x 49-44 on SFA Medium when a method involving the surface decontamination of cleistothecia with ethanol was used. This isolate was found to have a similar fingerprint as the *MAT1-2* parent (49-44) even though it was a *MAT1-1* isolate.

This finding is a very momentous one as this is the first evidence of actual genetic recombination in *A. terreus*. The previous report by Arabatzis and Velegraki (2012) showed evidence for formation of ascospores on morphological grounds but failed to present any direct evidence for recombination itself. However, the isolation of only one recombinant progeny was overall disappointing as it had been anticipated that far higher numbers would be obtained, as reported for other sexual *Aspergillus* species (O’Gorman *et al.*, 2009; Dyer and O’Gorman 2012; Swilaiman *et al.*, 2013). It is possible that very specific environmental conditions might be required to trigger germination of ascospores of *A. terreus* rather than the simple overnight incubation used in the present study. In parallel, similar challenges were encountered in obtaining clonal ascospore cultures of *A. clavatus* by Swilaiman (2013), but the differences in characteristics between ascospores and conidia were exploited to overcome them. The same approach was undertaken in this work with less success, and more investigations thus need to be performed to identify and exploit the unique characteristics of *A. terreus* ascospores in order to obtain clonal cultures. Nevertheless, further exploitation of the sexual cycle promises to be a promising method to generate genetically diverse strains of *A. terreus* with possible increased IA production, with a similar approach having been utilised for strain improvement of *Penicillium chrysogenum* for penicillin production (Böhm *et al.*, 2013).

Strain “T” was employed in an itaconic acid fermentation to determine the itaconic acid production capacity. The yield obtained was not very impressive at 3.71 g/L which indicates that strain “T” inherited the low itaconic acid producing capacity of parent 49-44.

### **6.5.3 Fermentation Optimisations**

#### ***6.5.3.1 Half-Factorial Screening of Factors***

A half-factorial experiment was designed and performed to screen for the most important factors that affect IA production by *A. terreus* in fermentations of RBDAAH, namely temperature, agitation, pH and inoculum size, with both actual concentrations (g/l) and efficiency of IA production measured. It was found that a particular set of conditions involving incubation at 33 °C, agitation of 180 rpm and pH 3.5 with a final inoculum concentration of  $1 \times 10^6$  spores/mL were the most conducive to improved itaconic acid yields,

resulting in 12.04 g/l with an efficiency of over 24 % of the theoretical maximum (**Table 6.11**). These **Figures** were higher than those obtained in previous fermentations (**Figures 4.12, 4.15, 4.16**) and even in fed-batch conditions (**Figure 4.20**). The conditions are very similar to those employed in previous reports where high itaconic acid yields were obtained from different feedstocks. For example, Kuenz *et al.* (2012) employed 33 °C, pH 3.1 and agitation of 120 rpm to attain IA concentrations of 86 - 91 g/l and this is one of the highest recorded yields in literature (See **Table 4.1** for more yields and conditions).

The yield of 24.25 % IA based on glucose consumed indicates that this process is also relatively efficient in glucose conversion. The Pareto chart (**Figure 6.14**) indicated that the agitation and inoculum size were not influential on the yields of the process. This contrasts with the findings of Riscaldati and co-workers (2000) who found that itaconic acid production was highly dependent on agitation/aeration rates. However, they employed very high agitation rates of 320 – 480 rpm (Riscaldati *et al.*, 2000) that were outside the scope and possibilities of this work. Given that high aeration rates are generally accepted as crucial to itaconic acid yields (Lockwood and Reeves, 1945; Nubel and Ratajak, 1962; Willke and Vorlop, 2001), the highest available rate of 200 rpm was deemed suitable for future experiments.

The main effects plot (**Figure 6.15**) indicated that increasing the temperature from 33 °C to 37 °C resulted in a decline in itaconic acid production while the reverse was true with pH. This is in agreement with the reports by several authors who obtained high IA yields at this temperature (**Table 4.1**). A low starting pH is necessary for itaconic acid production (Wilke and Vorlop, 2001) and several workers have achieved good itaconic acid yields with lower temperatures such as with sago starch at pH 2.0 (Dwiarty *et al.*, 2007) and on starch hydrolysates at pH 2.5 – 2.8 (Tsai *et al.*, 2001). However, in this work, low pH values were negatively correlated with itaconic acid yields as the values obtained at pH 2.7 ranged from 0.16 – 2.81 g/l which was considerably lower than values of over 12 g/l obtained under similar conditions with higher pH values.

The half-factorial screening experiment successfully narrowed down the four process parameters into two significant ones, temperature and pH, while indicating that agitation rate and inoculum size were not significant over the range tested. It was concluded that the inoculum size would be investigated over a wider range in the response surface method of optimisation, temperature with agitation held at 200 rpm. Inoculum size was included as the

third parameter to include in the main optimisations as it was the more significant of the two non-significant parameters and certain RSM analyses are more accurately performed when three variables are investigated.

#### **6.5.3.2 Optimisation by Response Surface Methodology**

A centred composite design (CCD), a response surface approach, was then created to optimise the significant variables in IA production namely temperature and pH, with inoculum size also investigated over a wider range. The RSM is a very useful statistics-based method which provides information about optimum levels of parameters and the interaction between variables while requiring minimal amounts of experiments (De Cássia Lacerda Brambilla Rodrigues *et al.*, 2012). In addition, it provides a polynomial model which can be used to predict the levels of variables that can be used to obtain a maximum response output. The design spanned wider ranges for the three parameters than investigated in the half-factorial screenings; particularly, it included lower temperatures and higher inoculum sizes and pH values as these were found to be more favourable for IA yield (**Table 6.8**).

The findings of the experiments indicated that the optimum conditions for IA production from red sorghum bran dilute acid hydrolysate with *A. terreus* 49-22 were 30 °C incubation temperature, pH 4.0 and one million spores/ml ( $\text{Log}_{10}$  6.0) which yielded up to 13.4 g/l (at an agitation of 200 rpm). This set of conditions are unique to this substrate-organism system, as a review of the conditions used in literature (**Table 4.1**) did not reveal the exact set being previously used to achieve high yields. However, the findings are in line with the general range of conditions used in the fermentation of various media and biomass hydrolysates. For instance, 30 °C was successfully employed by several workers (Jahim *et al.*, 2006; Juy *et al.*, 2010; Nikolay *et al.*, 2013); pH 4.5 was utilised by Vassilev *et al.* (2012); while most workers use  $1 \times 10^6$  spores /ml as a final inoculum concentration in the medium.

Analyses of variance showed that all the factors were significant at the 95.0% confidence level. The ANOVA **Table** partitions the variability in itaconic acid yield for each of the effects. It then tests the statistical significance of each effect. Notably, the effect of pH was the most significant with increasing pH favourable to yields, as was observed in the previous half-factorial experiments. The pH values below 3.5 may result in the denaturation of key enzymes. The effect of low pH was most starkly demonstrated in experiments 5 and 9 which only differed in pH values of 2.0 and 4.0 respectively, but resulted in yields of 0.3 versus

11.7 g/l itaconic acid, respectively. Meanwhile, inoculum size at the range tested was significant both alone and also in interaction with the other parameters (**Figure 6.17**). Increasing temperature initially increased IA yields, but this later became inhibitory, as was evident in the main effects plot (**Figure 6.16**). The inhibitory effect of high temperature is unlikely to be due to growth inhibition as *A. terreus* is a thermophile. Rather it is likely that the effect was due to decrease in activity of important enzymes in the IA pathway. A second order polynomial equation was fitted to the data obtained and was able to explain 89.13 % of the variability in itaconic acid production.

In terms of glucose utilisation, there was sometimes a trade-off in terms of actual IA concentration (g/l) and the efficiency of glucose conversion. For example, the relatively low yield of 2.2 g/l was achieved at a high efficiency of almost 50 % in experiment 1 whereas the highest yield of 13.5 g/l was achieved as a yield of only 28.3 % of the theoretical maximum in experiment 10 (**Table 6.15**). Overall though, the optimisations resulted in higher itaconic acid yield based on glucose consumed.

#### **6.5.3.3 OPTIMISATION VERIFICATION AND SCALE UP**

The model/equation could be applied to predict IA yields for a combination of conditions not performed, with reasonable accuracy especially within the range of the parameters it was modelled on (De Cássia Lacerda Brambilla Rodrigues et al., 2012). It predicted a set of optimum conditions to maximise IA production, but when these were implemented the yields were found to be significantly lower than obtained at the conditions observed in the previous CCD experiments. The latter conditions gave IA yields of 11.94 g/l with the high yield (%) based on theoretical maximum of 41.0 % glucose conversion, which was the highest combination of yield in g/l and as % of theoretical maximum. This was significantly higher than 7.9 g/l and 36.8 % obtained from the optimum conditions predicted from the model. Therefore it was concluded that the best conditions for IA production remained use of a 30 °C incubation temperature, pH 4.0 and inoculum density of  $1 \times 10^6$  spores/ml as observed from the CCD optimisation experiments. This disparity might have arisen because the model admittedly not 100 % accurate, although with an  $R^2$  value of 84.7 % the model was found to be sufficiently reliable to provide estimates over several other ranges (See **Appendix 7** for estimated values generated using this model).



The scale up experiments were then performed using these conditions and a very interesting increase was observed as yields rose to 13.9 g/l and 16.3 g/l as working fermentation volume increased from 25 ml to 200 ml and 500 ml, respectively. These increases are ascribed to better aeration due to the much larger headspace in the larger (2 L and 5 L) flasks. In addition, hydro-mechanical stress may be less and better distributed among the organisms in the larger flasks. The yields (%) of theoretical maximum also increased to 49.6 % and 46.1% respectively, both of which were the highest values obtained throughout this work and even greater improvements in itaconic acid values over those obtained from the optimisations alone. This increase during scale up contrasts with the decline in IA reported by Kuenz et al. (2012) from 90 g/l in 100 ml fermentation (in 250 ml flasks) to 86.2 g/l obtained in 10-L fermentation (performed in a 15-L bioreactor).

## 6.6 Summary and Conclusions

In this chapter, attempts were made to improve the yields obtained from red sorghum bran dilute acid hydrolysate used in itaconic acid production by *Aspergillus terreus* fermentation by three methods.

- The first method used was UV mutagenesis. A rationale screening method was employed based on tolerance to itaconic acid. 250 mutants were isolated and tested for IA production. Up to a 3.5 fold increase in yield was observed amongst the mutants. However, this improved performance could not be replicated in verification experiments and the work was painstaking, time-consuming and relatively random.
- The second method involved the use of sexual reproduction to generate genetic variation through recombination. A molecular diagnostic method was used to determine the mating types of both known sexually fertile or high IA producing isolates and these were crossed in all combinations. Hyphal masses containing putative cleistothecia, asci and ascospores were observed in several crosses, although most crosses were infertile. Some isolates were of particularly high fertility as judged by number of hyphal masses produced in crosses. Growth media type, temperature and gas exchange all influenced sexual fertility. Several attempts were made to isolate viable ascospore progeny, but only one recombinant ascospore-derived isolate could be verified when using RAPD-PCR banding pattern and mating-type as genetic markers. This exhibited the relatively low IA production characteristic of one parent.

- The third method involved optimisation of, the fermentation conditions using a combination of fractional factorial screening and a central composite design approach to response surface methodology. Fermentation parameters of temperature, pH, inoculum size and agitation were optimised. The level of agitation did not significantly affect IA yields, whereas higher temperatures decreased yields, while higher pH and inoculum sizes favoured itaconic acid production. A final yield of up to 13.7 g/l was obtained, which further increased during scale up experiments to up to 16.3 g/l in 500 ml fermentations. This represented an 85 % improvement in itaconic acid yield over the 8.8 g/l obtained with the same isolate of *A. terreus* in earlier work in the present study, while the efficiency of itaconic acid production based on glucose consumed improved from 24 % of the theoretical maximum to 49.6 % at the same time.

Overall, it was concluded that there was a low probability of obtaining high-yielding IA mutants via UV mutagenesis, that sexual reproduction offers the exciting potential to generate novel strains albeit that difficulties exist in obtaining ascospore offspring, and that it is feasible to optimise fermentation conditions to considerably improve itaconic acid production by *Aspergillus terreus*.

## Chapter 7: General Discussion

The overall aim of the research undertaken in this thesis was to attempt to utilise the bran waste which accumulates from the wet-milling of sorghum (*Sorghum bicolor* L. Moench) grains for the fermentative production of valuable industrial products. This crop is one of the most important cereals in the world with Nigeria being one of the world's largest producers producing about 7 MT in 2016/2017 ([www.worldsorghumproduction.com](http://www.worldsorghumproduction.com)). It is a drought-tolerant crop requiring minimal inputs for cultivation. It is primarily processed by wet-milling and the waste product accumulates in the environment or used as low-value animal feed.

Specifically, the research was aimed at determining whether itaconic acid and ethanol can be produced using sorghum bran as the sole substrate. Ethanol was chosen as a representative fuel because renewable ethanol is currently being used to replace petrol globally as a transportation fuel. This is because it is considered to be an environmentally beneficial energy source as its emissions on combustion are less toxic than those of petrol. Renewable ethanol production schemes currently utilise carbohydrates from food materials such as sucrose from sugarcane in Brazil and beet in France, and starch from maize in USA (Cardona, 2009; Demirbas, 2009), but this is not ideal as it competes with the food uses of these crops. Itaconic acid (IA) was selected as the renewable chemical to produce because it is a versatile chemical which finds applications in many industries and has been listed as one of the renewable chemicals with considerable potential (Ahmed El-Imam and Du, 2014; Werpy et al., 2004). It is also produced industrially from refined sugars such as pure glucose. However, its use is limited because of its high cost relative to the petroleum-based alternatives such as acrylic acid. One potential way to improve the economics of IA production is the use of cheaper substrates, given that IA might potentially be produced from hemicellulose (if a suitable organism is employed) at around a sixth of the market price (Van Heiningen 2006). In this chapter, data obtained from this study will now be discussed with a focus on each of the high-value products produced.

## 7.1 Chemical Composition of Sorghum Bran

The first objective of this study was to determine the composition of the brans obtained from the wet-milling process of two sorghum types (red and white), as no information was available on this in the scientific literature. It was discovered that the white and red brans appeared to contain all the major nutrient classes in adequate quantities to promote microbial growth. Specifically, it had a high starch content (50 – 53 %), which indicated that significant wastage is associated with the traditional milling and sieving process used in sorghum starch extraction. However, this high residual starch content meant it could be harnessed for bioconversion processes because starch provides an important carbon and energy source (Waites, 2009). In addition, the protein (15.6 % and 16.9 %), lipid (2.9 % and 3.7 %) and ash (1.8 % and 1.5 %) contents (for white and red brans, respectively) were also found to be relatively high. The brans were thus assessed to be suitable for the intended bioconversion uses and it was decided that bran hydrolysis would be employed for the generation of fermentable hydrolysates.

## 7.2 Generation of Hydrolysates

The hydrolysates were prepared by two methods, namely enzymatic hydrolysis and dilute acid hydrolysis. The enzymatic hydrolysis was performed using amylase and amyloglucosidase enzymes with a bran loading rate of 20 % over 24 h and yielded a final glucose concentration of 55 g/l and 61 g/l for WB and RB, respectively. Subsequent enzymatic digestions were terminated after eight hours.

With the acid hydrolysis, various conditions of catalyst (mineral acid) type and concentration, and residence time at a 5 % bran loading rate were investigated for the most efficient digestion process. The most abundant sugar liberated was glucose but trace amounts of xylose, arabinose and maltose were also observed. Digestion of WB with both  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$  resulted in approximately 70 % of the bran being converted to glucose corresponding to 27 – 35 g/l glucose. Acid concentration had a significant impact on glucose release, with 3 % concentrations of both acids liberating more sugars than 1 %, whereas change in residence time from 15 or 30 min had little impact. With RB,  $\text{H}_2\text{SO}_4$  was significantly the better catalyst, particularly at a 3 % level. Thus, it was decided that the use of a 3 %  $\text{H}_2\text{SO}_4$  digestion of both brans at 15 min was ideal for the production of a glucose-rich hydrolysate.

Sample hydrolysates namely white bran dilute acid hydrolysate (WBDAH), red bran dilute acid hydrolysate (RBDAH), white bran enzyme hydrolysate (WBEH) and red bran enzyme hydrolysate (RBEH) were produced which generally contained over 50 g/l glucose in solution from both methods. Investigations revealed that the levels of known fermentation inhibitors were relatively low in both hydrolysates, which was seen as a positive feature, although inhibitors were higher in the acid hydrolysates than the enzyme hydrolysates, particularly regarding HMF, furaldehyde and acetic acid.

### 7.3 Ethanol Production

Several ethanologenic yeast species were screened using spot plate method and phenotypic microarray (PM) techniques to determine the suitability of the hydrolysates to support yeast growth and metabolism. All media assayed were found to support growth in both aerobic and anaerobic conditions. It was evident that growth was enhanced by the higher glucose content of the enzyme hydrolysates as colonies were larger than those in the dilute acid hydrolysates. There was no difference in the growth patterns of the yeasts based on bran type. The PM analysis, to assess metabolic activity revealed that all the yeasts tested had similar growth profiles. *K. marxianus*, *S. cerevisiae* 1119 and *S. pastorianus* performed the best, and the effect of glucose content was again visible as yeasts grown on enzyme hydrolysates showed higher redox potential.

Anaerobic fermentations were carried out with *K. marxianus*, *S. pastorianus* and *S. cerevisiae* NCYC 2592 and it was observed that the highest weight loss was by *K. marxianus* and *S. cerevisiae* NCYC 2592 on RBEH. The ethanol yields as a function of the theoretical maximum were generally impressive, with all yeasts converting at least 63 % of the theoretical glucose, with the highest recorded conversion being 89.1 % for *K. marxianus* on RBEH (cf. 85.9 % on WBEH and 63.1 % on WBDAH). It was thus evident that the sorghum bran hydrolysates were suited for the fermentative production of ethanol with very high substrate utilisation efficiencies. *K. marxianus* was then used in a single fermentation on RBDAH and again high yields were observed with 7 g/l produced (equivalent to 63.5 % of theoretical maximum). This is the first report in literature of ethanol production from the bran from wet-milled sorghum.

## 7.4 Itaconic Acid Production

Next the two dilute acid hydrolysates (DAH) were employed in itaconic acid fermentation by *Aspergillus terreus*. Dilute acid hydrolysates were used because although the enzyme hydrolysates gave higher glucose yield, they were much more expensive to produce (results not shown), and the performances of the DAH in ethanol production assays were deemed to be sufficiently high. A total of 46 *A. terreus* isolates were screened in shake flask fermentation with a defined glucose medium previously identified as being highly suitable for IA production (Kuenz et al., 2012), and yields obtained that ranged from 0 – 44 g/l in a frequency distribution skewed towards lower values. Three of the best strains were selected and employed in a time course fermentation in RBDAH and WBDAH which showed, significantly, that itaconic acid can be produced from sorghum bran dilute acid hydrolysates. The maximum IA produced was 5.5 g/l by strain 49-22 on RBDAH. However, the IA was levels decreased after about four days, thought to be possibly due to an onward conversion to itatartaric acid.

The six best strains were then used to conduct 4-day fermentations and their yields relative to theoretical maximum estimated. Isolate 49-22 was consistently the best itaconic acid producer on both hydrolysates, producing IA yields of up to 22.9 % on RBDAH but only 15.1 % of theoretical maximum on WBDAH. However, yields were low compared to the 55 % conversion on defined glucose medium obtained in the defined medium fermentation. This was possibly thought to be due to the higher glucose content of the defined medium. Therefore, glucose enrichment time-course fermentations were performed using RBDAH and it was observed that IA yields did indeed increase marginally to 10.1 g/l (with 49-5), but this increase was not commensurate with the glucose consumed which meant an efficiency of just 10.2 % of theoretical maximum. Fed-batch fermentations were performed which also increased output (from 5.2 to 9.0 g/l). In all instances, mycelial mass formation was significantly higher in the hydrolysates than in the defined medium, and this appeared to partly explain the lower IA yields as carbon is preferentially diverted to biomass formation at the expense of product formation.

Overall it was therefore possible to demonstrate that sorghum bran provides a suitable feedstock for ethanol and itaconic acid production, with more work being required to further improve the economic viability of the process, particularly on an industrial scale. Both products were successfully produced without the need for complex equipment or additional

nutrients and the bioconversions were completed in short times. This is the first report in literature of the use of sorghum bran for itaconic acid production.

## **7.5 Hydrolysate Purification/Detoxification**

In the case of IA production, it was assumed that the hydrolysates contained certain components that favoured mycelial mass formation over secondary metabolism and IA production i.e. these components acted as inhibitors of IA fermentations. Therefore, several conditioning treatments were performed in order to purify the RBDH, to determine whether IA yield could be increased. These treatments included 13 physical and chemical methods classically employed in the detoxification of biomass hydrolysates for ethanol fermentation (Palmqvist et al., 1996). To the best of the researcher's knowledge, this represented the first attempt to purify (detoxify) biomass hydrolysates as a means to successfully improve the outcome of itaconic acid fermentation with *A. terreus*.

The alkaline treatment processes were very efficient at reducing total phenolic compounds with detannification removing 73.8 %, and overliming removing over half. Detannification also removed 75 %, of the condensed tannin, and the anion exchanger Dowex 66 removed over 90 %. However, phosphorus removal using a bead treatment was not successful. Six methods resulted in glucose loss, notably overliming and the NaOH detannification process. The purified hydrolysates were used in fermentations with strain 49-22, and slightly higher IA yields were obtained from the activated charcoal and overliming treatments compared to the RBDH control fermentation. However, the increases were fairly small, and purification of the hydrolysates with other methods resulted in lower itaconic acid yields despite the fact that all purified hydrolysates supported mycelial formation.

## **7.6 Strain Improvement and Process Optimisation**

It was apparent from work in the present study that although the hydrolysates appeared suitable for ethanol fermentation, they were not producing optimum amounts for itaconic acid production. Therefore attempts were made to both develop strains with enhanced genetic abilities for production of valuable compounds, given that production from wild type organisms is often not economical (Han and Parekh, 2005), and increase the efficiency of the fermentation process.

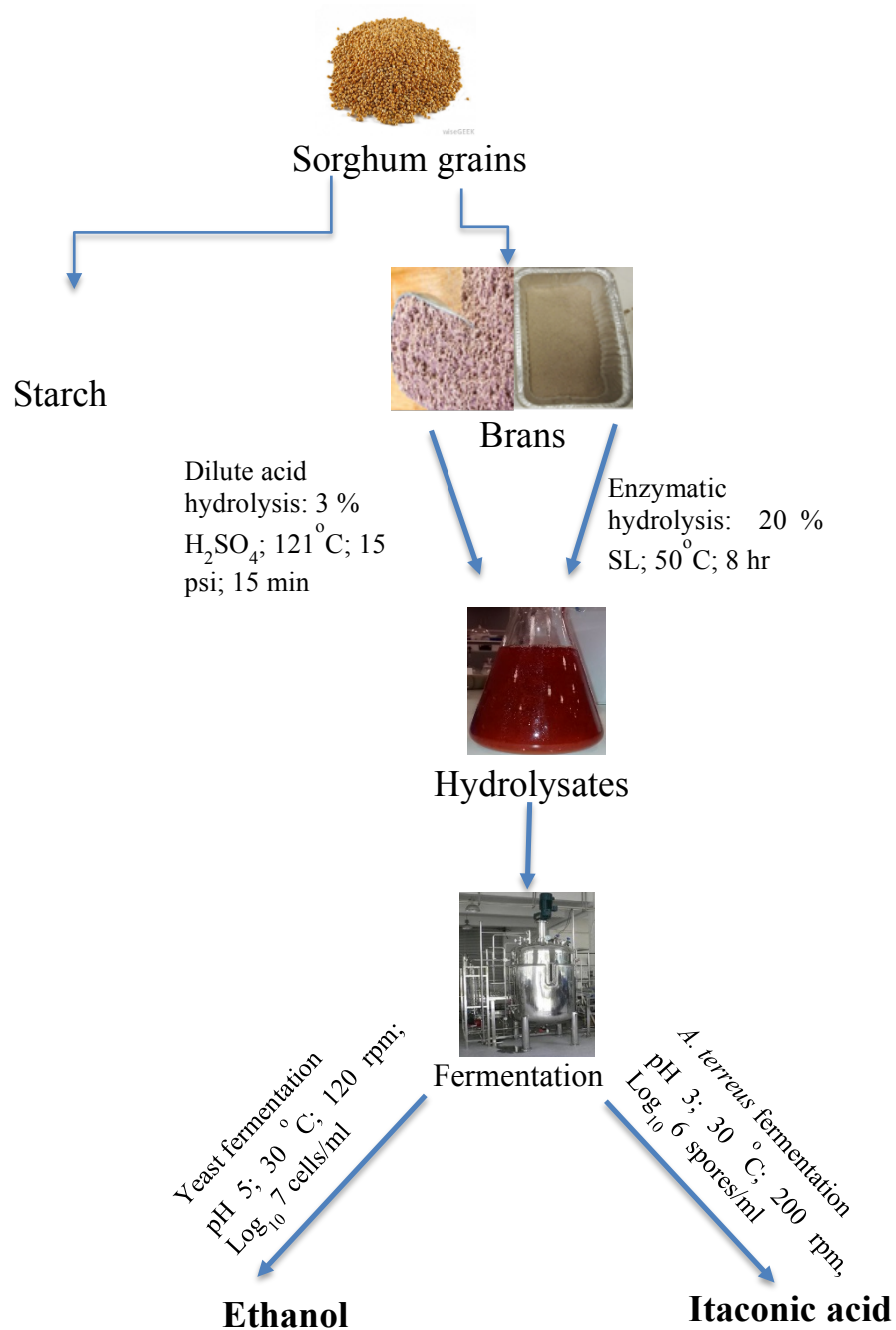
Two methods were employed for strain improvement in attempts to yield progeny that can utilise the hydrolysates to produce high amounts of IA efficiently. Firstly, the two best strains for RBDAH fermentation (49-5 and 49-22) were exposed to UV mutagenesis and rationale screening method used (Yahiro et al., 1995). Although it was possible to select mutants with apparent major increases in IA yield, unfortunately these increases could not be replicated in subsequent fermentations. Secondly, sexual crossing was investigated as a means to generate progeny with genome-wide recombination that might have enhanced IA fermentation characteristics. Crosses were set up under a variety of environmental conditions using isolates of known sexual fertility (Arabatzis and Velegraki, 2013) and also higher IA yield. Excitingly, hyphal masses with apparent cleistothecia and ascospores were produced in some crosses, particularly with moderate levels of gas exchange. A novel sorghum flour medium was found to be considerably better at inducing sexual reproduction than the previous ‘best’ medium for sex in *A. terreus*, that of Arabatzis and Velegraki (2013). However, many isolates failed to produce cleistothecia. Furthermore, efforts to obtain clonal ascospore suspensions were not successful, with only one putative recombinant isolate obtained after ethanol treatment of contaminating conidia. This isolate was identified from a combination of RAPD-PCR fingerprinting and *MAT* typing to be a recombinant progeny, which is the first report of genetic evidence for sexual recombination in *A. terreus* given that the previous report of Arabatzis and Velegraki (2013) only reported sex based on morphological evidence. Indeed, it is tempting to speculate that these authors also probably encountered difficulties in obtaining viable ascospore progeny. The recombinant strain disappointingly did not exhibit any increased IA fermentation abilities.

Meanwhile, attempts were made to optimise the fermentation processes using the central composite design (CCD) method of response surface methodology (RSM). A half-factorial screening method was employed to identify important parameters for itaconic acid fermentation and these were then investigated over various ranges in the RSM. The yields in g/l and the efficiency of the process in terms of itaconic acid produced (%) based on theoretical maximum possible from the glucose consumed were then determined. The half-factorial experiments revealed a combination of conditions (33 °C, pH 3.5 and 180 rpm) which resulted in over 12 g/l IA equivalent to over 24 % of the theoretical maximum. It also indicated that temperature and pH were the most important parameters, while agitation and inoculum sizes were not very important in the limited range evaluated. Subsequent RSM experiments at constant agitation verified that the three parameters were statistically



significant with inoculum size also positively correlating with IA yields. The RSM experiments showed that low pH (below 3.0) and high temperatures were detrimental to IA production. They also showed that the best conditions for red sorghum bran dilute acid hydrolysate fermentation with *A. terreus* 49-22 were 30 °C, pH 4.0 and  $1 \times 10^6$  spores with yields up to 13.5 g/l (with agitation of 200 rpm). The highest efficiency of IA production, about 50 % of theoretical maximum was observed in these experiments (albeit corresponding to low actual amounts) while the 13.5 g/l best yields were also equivalent to 28 % of theoretical maximum, also an improvement over the half-factorial results. These conditions were verified to produce reproducible high yields in subsequent experiments. A prediction model with a  $R^2$  of 89.1 % was generated from the RSM, which was considered fairly accurate.

An overall summary for the fermentation process developed in the present study is illustrated in **Figure 7.1** below.



**Figure 7.1:** Flowchart summarising the bioconversion of sorghum bran into value-added chemicals.

## 7.7: Future work

The present study has provided various leads for future work related to possible future commercial use of sorghum bran hydrolysates in the production of value-added products. This is in the context of bioethanol and itaconic acid production and beyond.

- The enzymatic hydrolysis of the sorghum brans needs to be investigated in greater detail and the optimum conditions determined for possible increase in glucose release.
- Mutagenesis of the *A. terreus* strains should be attempted with other mutagens such as chemicals and higher numbers of progeny will need to be screened.
- Modern methods of genetic manipulation might be considered for direct genetic alteration of high yielding strains, particularly where enzymes linked to production pathways are known. For example, IA is thought to be produced in a pathway via aconitase and *cis*-aconitate decarboxylase and the genes responsible have been identified (Dwiarty *et al.*, 2002; Kanamasa *et al.*, 2008). However, such approaches require specialist skills and equipment.
- Itaconic acid and ethanol fermentation will be performed in greater detail with WBDH to determine its potential for improvement.
- It will be interesting to determine the component of the sorghum flour that makes SFA favourable for sexual reproduction; particular to investigate the composition of ions, sterols and phytochemicals that are unique to it. SFA might prove to be a very valuable new medium to try to induce the sexual reproduction in other supposedly asexual species with no known sexual cycle.
- The process for the isolation of *A. terreus* ascospores needs to be improved significantly. This can be done by investigating and exploiting any inherent differences in the characteristics of ascospores and conidiospores, in order to get ascospore-rich suspensions for further characterisation. Particular germination triggers might be required for ascospores such as high pressure and temperature?
- It is important to investigate the effect of further scale-up for example up to 1.5 L and 10 L working volume on both ethanol and itaconic acid yields and efficiency.
- Lastly, the feasibility of designing a whole-crop biorefinery to include the industrial production of sorghum starch, and the utilisation of the bran for bioconversion processes such as described in this thesis will be investigated. This will also include cost benefit analyses, life cycle analysis and environmental impact assessment of the process.

## **7.8 Conclusion**

Key aims of this work have been achieved. The chemical composition of sorghum bran was determined and optimum pretreatment conditions for hydrolysate production successfully elucidated. The hydrolysates were successfully employed in fermentation to produce ethanol and itaconic acid. The production of improved strains for IA production was only marginally successful, but potential progeny were identified by sexual reproduction. Lastly, the fermentation was scaled up successfully with improved yields, and stable yields were attained under optimum conditions. The processes have been optimised using the least expensive alternatives possible, but the exact economic competitiveness of the various processes still need to be determined.

It is believed that the findings of this work have highlighted hitherto unknown possible biotechnological applications for sorghum bran and the sorghum crop itself.

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## Appendices

**Appendix 1:** Isolates of *Aspergillus terreus* employed in this work and details of their source and mating types.

STRAIN	OTHER NAMES	MATING TYPE	MAT SOURCE*	STRAIN SOURCE†
49-1	NRRL 255; EF 66958	<i>MAT 1-2</i>	THIS WORK	N/A
49-2	T6	<i>MAT 1-2</i>	THIS WORK	N/A
49-3	Uab 29	<i>MAT 1-1</i>	THIS WORK	Clinical
49-4	IBT 16744	<i>MAT 1-1</i>	EAGLE, 2009	Environmental
49-5	IBT 6450	<i>MAT 1-2</i>	EAGLE, 2009; THIS WORK	Environmental
49-6	UAB 11	<i>MAT 1-2</i>	THIS WORK	Clinical
49-7	T 16	<i>MAT 1-1</i>	BDUN*	N/A
49-8	T 37	<i>MAT 1-1</i>	BDUN	N/A
49-9	T 120	<i>MAT 1-2</i>	BDUN	N/A
49-10	ATCC 20542	<i>MAT 1-1</i>	THIS WORK	N/A
49-11	ATCC 20542	<i>MAT 1-1</i>	BDUN	Soil
49-12	CBS 2B7	<i>MAT 1-1</i>	THIS WORK	N/A
49-13	DTO-005-D1; CBS 811.96	<i>MAT 1-2</i>	THIS WORK	N/A
49-14	CBS 619	<i>MAT 1-2</i>	THIS WORK	Soil
49-15	CBS 8G3	<i>MAT 1-1</i>	THIS WORK	Blended almond pits
49-16	CBS 8G5	NR		White soy beans
49-17	DTO-011-F1	<i>MAT 1-1</i>	THIS WORK	Indoor air
49-18	CBS 17A1	<i>MAT 1-2</i>	THIS WORK	Laboratory medium
49-19	CBS 24A4	<i>MAT 1-2</i>	THIS WORK	Sugar silo door
49-20	CBS 601.65	<i>MAT 1-1</i>	THIS WORK	Connecticut soil
49-21	CBS 594.65	<i>MAT 1-2</i>	THIS WORK	Soil
49-22	DTO-025-D3; CBS 116.46	<i>MAT 1-1</i>	THIS WORK	Soil



49-23	CBS 125.38	<i>MAT 1-1</i>	THIS WORK	N/A
49-24	DTO-025-D6; CBS 377.64	<i>MAT 1-2</i>	THIS WORK	Haversack
49-25	CBS 383.75	<i>MAT 1-2</i>	THIS WORK	Soil
49-26	DTO-032-B9	<i>MAT 1-2</i>	THIS WORK	Bakery
49-27	IBT 26915	<i>MAT 1-2</i>	EAGLE, 2009	N/A
49-28	IBT 24859	<i>MAT 1-1</i>	THIS WORK	N/A
49-29	IBT 21125	<i>MAT 1-1</i>	EAGLE, 2009	Stored paddy
49-30	IBT 12713	<i>MAT 1-1</i>	EAGLE, 2009	Kangaroo rat
49-31	IBT 16745	<i>MAT 1-1</i>	EAGLE, 2009	Soil
49-32	DTO-039-F7; CBS N116757	<i>MAT 1-2</i>	THIS WORK	Wall paint basement
49-33	DTO-Tao-5H3	<i>MAT 1-2</i>	THIS WORK	Coffee bean
49-34	CBS 469.81	<i>MAT 1-1</i>	EAGLE, 2009	Cardiac valve
49-35	DTO-138-F2; CBS 116686	<i>MAT 1-1</i>	THIS WORK	Milled rice
49-36	DTO-138-F3; CBS 116878	<i>MAT 1-2</i>	THIS WORK	Soil
49-37	DTO-138-B3; CBS 134.60	<i>MAT 1-2</i>	THIS WORK	Cotton
49-38	DTO-138- B4	<i>MAT 1-2</i>	THIS WORK	Cotton
49-39	N/A	<i>MAT 1-1</i>	THIS WORK	N/A
49-40	UOA 10536	<i>MAT 1-1</i>	Arabatzi & Velegaki, 2012	Urine
49-41	Env 49	<i>MAT 1-1</i>	Arabatzi & Velegaki, 2012	Archaeological specimen
49-42	UOA 3706	<i>MAT 1-1</i>	Arabatzi & Velegaki, 2012	Abscess
49-43	UOA 9927	<i>MAT 1-1</i>	Arabatzi & Velegaki, 2012	Bronchoalveolar lavage
49-44	UOA 12626A	<i>MAT 1-2</i>	Arabatzi & Velegaki, 2012	Spoilt pre-cooked pasta
49-45	NRRL 1960; DSM 826	<i>MAT 1-1</i>	THIS WORK	Soil
49-46	SBUG8GG	<i>MAT 1-2</i>	THIS WORK	N/A

\* Source of *MAT* type information; the *MAT* type of some strains were determined prior to this work and the references are shown.

† Sourced from Swilaiman (2013); Arabatzis and Velegaki (2012).

N/A = Not available; NR Not reactive with *MAT* primers used.

## Appendix 2: Yeast strains employed in ethanol fermentations

STRAIN	OTHER NAMES	SOURCE
<i>Saccharomyces cerevisiae</i> NCYC 2592	CBS 1200; ATCC4126	Distiller's production strain
<i>Scheffersomyces stipitis</i>		
<i>Wickerhamomyces anomalous</i>		
<i>Kluyveromyces marxianus</i>		
<i>S. cerevisiae</i> NCYC 1383	ATCC 44774; DBY 747	Genetically defined strain
<i>S. pastorianus</i>		
<i>S. cerevisiae</i> NCYC 1119	N/A	British ale strain
<i>Candida arabinofermentas</i> NCYC 2916	CBS 8468; NRRL YB-2248	Insect frass USA

**Appendix 3:** Itaconic acid yields obtained from the fermentation of five *Aspergillus terreus* isolates on RBDAH and defined glucose media of different concentrations. Values are averages of triplicates with the standard deviations.

GLUCOSE CONTENT STRAIN	DEFINED MEDIUM				RBDAH			
	ITACONIC ACID (g/L)		GLUCOSE CONSUMED (g/L)		ITACONIC ACID (g/L)		GLUCOSE CONSUMED (g/L)	
	51.6	120	51.6	120	51.6	120	51.6	120
49-1	13.22 ± 1.39	31.54 ± 0.55	51.47	114.37	4.19 ± 0.8	5.28 ± 0.83	51.6	114.37
49-5	19.86 ± 1.44	45.07 ± 2.01	49.12	110.27	4.52 ± 1.35	7.63 ± 2.47	50.29	110.27
49-22	16.93 ± 1.35	42.18 ± 2.66	50.79	104.82	8.84 ± 3.33	10.98 ± 1.57	51.08	104.82
49-43	18.22 ± 2.30	33.19 ± 0.94	51.6	97.43	4.27 ± 1.11	6.13 ± 1.33	51.52	97.43
49-45	19.93 ± 1.43	42.85 ± 2.08	51.15	103.75	4.17 ± 0.19	6.86 ± 0.27	51.42	103.75

**Appendix 4:** Multiple Range Tests for itaconic acid yield (%) by hydrolysate purification type. Method: 95.0 percent LSD

<i>Level</i>	<i>Count</i>	<i>Mean</i>	<i>Homogeneous Groups</i>
DOWEX 50WX2	3	5.22333	X
AMBERLITE XAD	3	6.44333	X
PHOSPHORUS REMOVAL	3	6.59	X
AMBERLITE 2	3	9.41333	X
DOWEX 50WX8	3	9.94667	X
LIME NEUTRALIZATION	3	10.5267	X
UF PERMEATE	3	12.0433	X
DOWEX 66	3	18.5467	X
RBDAH	3	21.98	X
DETANNIFICATION	3	22.0567	X
OVERLIMED RBDAH	3	22.08	X
ACTIVATED CHARCOAL	3	24.6	X

<b>Contrast</b>	<b>Sig.</b>	<b>Difference</b>	<b>+/- Limits</b>
ACTIVATED CHAR - AMBERLITE 2	*	15.1867	0.52852
ACTIVATED CHAR - AMBERLITE XAD	*	18.1567	0.52852
ACTIVATED CHAR - DETANNIFICATIO	*	2.54333	0.52852
ACTIVATED CHAR - DOWEX 50WX2	*	19.3767	0.52852
ACTIVATED CHAR - DOWEX 50WX8	*	14.6533	0.52852
ACTIVATED CHAR - DOWEX 66	*	6.05333	0.52852
ACTIVATED CHAR - LIME NEUTRALIZTION	*	14.0733	0.52852
ACTIVATED CHAR - OVERLIMED	*	2.52	0.52852
ACTIVATED CHAR - PHOSPHORUS REM	*	18.01	0.52852
ACTIVATED CHAR - RBDAH	*	2.62	0.52852
ACTIVATED CHAR - UF PERMEATE	*	12.5567	0.52852
AMBERLITE 2 - AMBERLITE XAD 16	*	2.97	0.52852
AMBERLITE 2 - DETANNIFICATION	*	-12.643	0.52852
AMBERLITE 2 - DOWEX 50WX2	*	4.19	0.52852
AMBERLITE 2 - DOWEX 50WX8	*	-0.5333	0.52852
AMBERLITE 2 - DOWEX 66	*	-9.1333	0.52852
AMBERLITE 2 - LIME NEUTRALIZATION	*	-1.1133	0.52852
AMBERLITE 2 - OVERLIMED	*	-12.667	0.52852
AMBERLITE 2 - PHOSPHORUS REM	*	2.82333	0.52852
AMBERLITE 2 - RBDAH	*	-12.567	0.52852
AMBERLITE 2 - UF PERMEATE	*	-2.63	0.52852
AMBERLITE XAD - DETANNIFICATIO	*	-15.613	0.52852
AMBERLITE XAD - DOWEX 50WX2	*	1.22	0.52852
AMBERLITE XAD - DOWEX 50WX8	*	-3.5033	0.52852
AMBERLITE XAD - DOWEX 66	*	-12.103	0.52852

AMBERLITE XAD - LIME NEUTRALIZ	*	-4.0833	0.52852
AMBERLITE XAD - OVERLIMED	*	-15.637	0.52852
AMBERLITE XAD - PHOSPHORUS REM		-0.1467	0.52852
AMBERLITE XAD - RBDHAH	*	-15.537	0.52852
AMBERLITE XAD - UF PERMEATE	*	-5.6	0.52852
DETANNIFICATIO - DOWEX 50WX2	*	16.8333	0.52852
DETANNIFICATIO - DOWEX 50WX8	*	12.11	0.52852
DETANNIFICATIO - DOWEX 66	*	3.51	0.52852
DETANNIFICATIO - LIME NEUTRALIZ	*	11.53	0.52852
DETANNIFICATIO - OVERLIMED		-0.0233	0.52852
DETANNIFICATIO - PHOSPHORUS REM	*	15.4667	0.52852
DETANNIFICATIO - RBDHAH		0.07667	0.52852
DETANNIFICATIO - UF PERMEATE	*	10.0133	0.52852
DOWEX 50WX2 - DOWEX 50WX8	*	-4.7233	0.52852
DOWEX 50WX2 - DOWEX 66	*	-13.323	0.52852
DOWEX 50WX2 - LIME NEUTRALIZ	*	-5.3033	0.52852
DOWEX 50WX2 - OVERLIMED	*	-16.857	0.52852
DOWEX 50WX2 - PHOSPHORUS REM	*	-1.3667	0.52852
DOWEX 50WX2 - RBDHAH	*	-16.757	0.52852
DOWEX 50WX2 - UF PERMEATE	*	-6.82	0.52852
DOWEX 50WX8 - DOWEX 66	*	-8.6	0.52852
DOWEX 50WX8 - LIME NEUTRALIZ	*	-0.58	0.52852
DOWEX 50WX8 - OVERLIMED	*	-12.133	0.52852
DOWEX 50WX8 - PHOSPHORUS REM	*	3.35667	0.52852
DOWEX 50WX8 - RBDHAH	*	-12.033	0.52852
DOWEX 50WX8 - UF PERMEATE	*	-2.0967	0.52852
DOWEX 66 - LIME NEUTRALIZ	*	8.02	0.52852
DOWEX 66 - OVERLIMED	*	-3.5333	0.52852
DOWEX 66 - PHOSPHORUS REM	*	11.9567	0.52852
DOWEX 66 - RBDHAH	*	-3.4333	0.52852
DOWEX 66 - UF PERMEATE	*	6.50333	0.52852
LIME NEUTRALIZ - OVERLIMED	*	-11.553	0.52852
LIME NEUTRALIZ - PHOSPHORUS REM	*	3.93667	0.52852
LIME NEUTRALIZ - RBDHAH	*	-11.453	0.52852
LIME NEUTRALIZ - UF PERMEATE	*	-1.5167	0.52852
OVERLIMED - PHOSPHORUS REM	*	15.49	0.52852
OVERLIMED - RBDHAH		0.1	0.52852
OVERLIMED - UF PERMEATE	*	10.0367	0.52852
PHOSPHORUS REM - RBDHAH	*	-15.39	0.52852
PHOSPHORUS REM - UF PERMEATE	*	-5.4533	0.52852
RBDHAH - UF PERMEATE	*	9.93667	0.52852

\* denotes a statistically significant difference.

Note: This **Table** applies a multiple comparison procedure to determine which means are significantly different from which others. The top half shows 9 homogenous groups which are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 62 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure.

**Appendix 5:** Schedule of crosses

<b>CROSS</b>	<b>S/NO</b>	<b>MEDIUM</b>	<b>SEALING?</b>	<b>NO OF HYPHAL MASSES (37 °C)</b>	<b>S/NO</b>	<b>NO OF HYPHAL MASSES (32 °C)</b>
<b>49-22 X 49-1</b>						
	<b>1</b>	<b>MCA</b>	SEALED	1	<b>145</b>	0
	<b>2</b>		SEALED	0	<b>146</b>	0
	<b>3</b>		BAGGED	0	<b>147</b>	0
	<b>4</b>		BAGGED	0	<b>148</b>	0
	<b>5</b>	<b>OOA</b>	SEALED	0	<b>149</b>	0
	<b>6</b>		SEALED	0	<b>150</b>	0
	<b>7</b>		BAGGED	0	<b>151</b>	0
	<b>8</b>		BAGGED	0	<b>152</b>	0
	<b>9</b>	<b>SFA</b>	SEALED	0	<b>153</b>	0
	<b>10</b>		SEALED	0	<b>154</b>	0
	<b>11</b>		BAGGED	0	<b>155</b>	0
	<b>12</b>		BAGGED	1	<b>156</b>	0
<b>49-40 X 49-1</b>	<b>13</b>	<b>MCA</b>	SEALED	0	<b>157</b>	0
	<b>14</b>		SEALED	0	<b>158</b>	0
	<b>15</b>		BAGGED	0	<b>159</b>	0
	<b>16</b>		BAGGED	0	<b>160</b>	0
	<b>17</b>	<b>OOA</b>	SEALED	0	<b>161</b>	0
	<b>18</b>		SEALED	0	<b>162</b>	0
	<b>19</b>		BAGGED	0	<b>163</b>	0
	<b>20</b>		BAGGED	0	<b>164</b>	0
	<b>21</b>	<b>SFA</b>	SEALED	0	<b>165</b>	0
	<b>22</b>		SEALED	6	<b>166</b>	0
	<b>23</b>		BAGGED	0	<b>167</b>	5
	<b>24</b>		BAGGED	6	<b>168</b>	1
<b>49-43 X 49-1</b>	<b>25</b>	<b>MCA</b>	SEALED	0	<b>169</b>	0
	<b>26</b>		SEALED	0	<b>170</b>	0
	<b>27</b>		BAGGED	0	<b>171</b>	0
	<b>28</b>		BAGGED	0	<b>172</b>	0
	<b>29</b>	<b>OOA</b>	SEALED	0	<b>173</b>	0
	<b>30</b>		SEALED	0	<b>174</b>	0
	<b>31</b>		BAGGED	0	<b>175</b>	0
	<b>32</b>		BAGGED	0	<b>176</b>	0
	<b>33</b>	<b>SFA</b>	SEALED	1	<b>177</b>	0
	<b>34</b>		SEALED	0	<b>178</b>	0
	<b>35</b>		BAGGED	1	<b>179</b>	0
	<b>36</b>		BAGGED	0	<b>180</b>	0
<b>49-45 X 49-1</b>	<b>37</b>	<b>MCA</b>	SEALED	0	<b>181</b>	0
	<b>38</b>		SEALED	0	<b>182</b>	0
	<b>39</b>		BAGGED	0	<b>183</b>	0
	<b>40</b>		BAGGED	0	<b>184</b>	0
	<b>41</b>	<b>OOA</b>	SEALED	0	<b>185</b>	0
	<b>42</b>		SEALED	0	<b>186</b>	0
	<b>43</b>		BAGGED	0	<b>187</b>	0

	44		BAGGED	0	188	0
	45	SFA	SEALED	0	189	0
	46		SEALED	0	190	0
	47		BAGGED	0	191	0
	48		BAGGED	0	192	0
49-22 X 49-5	49	MCA	SEALED	0	193	0
	50		SEALED	0	194	0
	51		BAGGED	X*	195	0
	52		BAGGED	X*	196	0
	53	OOA	SEALED	0	197	0
	54		SEALED	0	198	0
	55		BAGGED	0	199	0
	56		BAGGED	0	200	0
	57	SFA	SEALED	0	201	0
	58		SEALED	0	202	0
	59		BAGGED	0	203	0
	60		BAGGED	0	204	0
49-40 X 49-5	61	MCA	SEALED	2	205	0
	62		SEALED	2	206	0
	63		BAGGED	1	207	0
	64		BAGGED	0	208	0
	65	OOA	SEALED	1	209	0
	66		SEALED	6	210	0
	67		BAGGED	0	211	0
	68		BAGGED	0	212	0
	69	SFA	SEALED	0	213	0
	70		SEALED	0	214	0
	71		BAGGED	0	215	0
	72		BAGGED	0	216	0
49-43 X 49-5	73	MCA	SEALED	0	217	0
	74		SEALED	0	218	0
	75		BAGGED	0	219	0
	76		BAGGED	0	220	0
	77	OOA	SEALED	11	221	0
	78		SEALED	2	222	0
	79		BAGGED	2	223	0
	80		BAGGED	4	224	0
	81	SFA	SEALED	0	225	7
	82		SEALED	0	226	6
	83		BAGGED	0	227	0
	84		BAGGED	0	228	0
49-45 X 49-5	85	MCA	SEALED	0	229	0
	86		SEALED	2	230	0
	87		BAGGED	3	231	0
	88		BAGGED	0	232	0
	89	OOA	SEALED	0	233	0
	90		SEALED	2	234	0
	91		BAGGED	3	235	0

	<b>92</b>		BAGGED	0	<b>236</b>	0
	<b>93</b>	<b>SFA</b>	SEALED	0	<b>237</b>	0
	<b>94</b>		SEALED	2	<b>238</b>	0
	<b>95</b>		BAGGED	0	<b>239</b>	0
	<b>96</b>		BAGGED	0	<b>240</b>	0
<b>49-22 X 49-44</b>	<b>97</b>	<b>MCA</b>	SEALED	0	<b>241</b>	8
	<b>98</b>		SEALED	0	<b>242</b>	6
	<b>99</b>		BAGGED	0	<b>243</b>	0
	<b>100</b>		BAGGED	0	<b>244</b>	0
	<b>101</b>	<b>OOA</b>	SEALED	0	<b>245</b>	0
	<b>102</b>		SEALED	1	<b>246</b>	0
	<b>103</b>		BAGGED	1	<b>247</b>	3
	<b>104</b>		BAGGED	2	<b>248</b>	0
	<b>105</b>	<b>SFA</b>	SEALED	113	<b>249</b>	61
	<b>106</b>		SEALED	120	<b>250</b>	52
<b>49-40 X 49-44</b>	<b>107</b>		BAGGED	36	<b>251</b>	45
	<b>108</b>		BAGGED	83	<b>252</b>	80
	<b>109</b>	<b>MCA</b>	SEALED	0	<b>253</b>	20
	<b>110</b>		SEALED	2	<b>254</b>	17
	<b>111</b>		BAGGED	9	<b>255</b>	1
	<b>112</b>		BAGGED	9	<b>256</b>	1
	<b>113</b>	<b>OOA</b>	SEALED	8	<b>257</b>	31
	<b>114</b>		SEALED	0	<b>258</b>	23
	<b>115</b>		BAGGED	5	<b>259</b>	95
	<b>116</b>		BAGGED	8	<b>260</b>	92
<b>49-43 X 49-44</b>	<b>117</b>	<b>SFA</b>	SEALED	34	<b>261</b>	16
	<b>118</b>		SEALED	23	<b>262</b>	11
	<b>119</b>		BAGGED	84	<b>263</b>	41
	<b>120</b>		BAGGED	80	<b>264</b>	48
	<b>121</b>	<b>MCA</b>	SEALED	0	<b>265</b>	11
	<b>122</b>		SEALED	0	<b>266</b>	13
	<b>123</b>		BAGGED	7	<b>267</b>	10
	<b>124</b>		BAGGED	13	<b>268</b>	14
	<b>125</b>	<b>OOA</b>	SEALED	0	<b>269</b>	2
	<b>126</b>		SEALED	0	<b>270</b>	1
<b>49-45 X 49-44</b>	<b>127</b>		BAGGED	11	<b>271</b>	0
	<b>128</b>		BAGGED	12	<b>272</b>	1
	<b>129</b>	<b>SFA</b>	SEALED	26	<b>273</b>	41
	<b>130</b>		SEALED	15	<b>274</b>	34
	<b>131</b>		BAGGED	23	<b>275</b>	20
	<b>132</b>		BAGGED	20	<b>276</b>	15
	<b>133</b>	<b>MCA</b>	SEALED	3	<b>277</b>	0
	<b>134</b>		SEALED	2	<b>278</b>	0
	<b>135</b>		BAGGED	19	<b>279</b>	0
	<b>136</b>		BAGGED	21	<b>280</b>	0
<b>49-45 X 49-44</b>	<b>137</b>	<b>OOA</b>	SEALED	1	<b>281</b>	4
	<b>138</b>		SEALED	10	<b>282</b>	5
	<b>139</b>		BAGGED	26	<b>283</b>	19

	<b>140</b>		BAGGED	17	<b>284</b>	15
	<b>141</b>	<b>SFA</b>	SEALED	30	<b>285</b>	22
	<b>142</b>		SEALED	23	<b>286</b>	25
	<b>143</b>		BAGGED	27	<b>287</b>	31
	<b>144</b>		BAGGED	31	<b>288</b>	38

X denotes the formation of large number of unidentified small aggregates along the barrage region; these are not tallied in the counts of hyphal masses for the cross

MCA = Mixed cereal agar; OOA = Odum's oatmeal agar; SFA = Sorghum flour agar



**Appendix 6:** Itaconic acid yields of all strains obtained from the mutation of strains 49-5 and 49-22

MUTANT NUMBER	ITACONIC ACID YIELD g/L	MUTANT NUMBER	ITACONIC ACID YIELD g/L	MUTANT NUMBER	ITACONIC ACID YIELD g/L
M1	16.4	M34	2.38	M67	2.58
M2	2.91	M35	2.85	M68	2.73
M3	0.41	M36	3.21	M69	2.38
M4	0.54	M37	2.66	M70	2.72
M5	0.45	M38	2.26	M71	2.64
M6	3.12	M39	2.64	M72	3.66
M7	3.06	M40	2.2	M73	3.25
M8	0.99	M41	2.3	M74	3.24
M9	3.03	M42	1.95	M75	2.64
M10	2.9	M43	2.62	M76	2.39
M11	3	M44	2.62	M77	2.19
M12	3.19	M45	3.07	M78	3.4
M13	3.94	M46	2.16	M79	2.46
M14	2.99	M47	2.45	M80	2.34
M15	1.22	M48	2.97	M81	2.88
M16	2.62	M49	4.82	M82	2.79
M17	3	M50	4.19	M83	2.66
M18	2.62	M51	3.92	M84	3.27
M19	2.92	M52	3.31	M85	0.44
M20	3.47	M53	2.47	M86	1.61
M21	3.31	M54	2.94	M87	2.14
M22	3.2	M55	2.25	M88	2.6
M23	3.36	M56	3.18	M89	2.31
M24	3.02	M57	3.27	M90	3.14
M25	4.71	M58	3.01	M91	2.53
M26	0.54	M59	2.59	M111	2.29
M27	4.48	M60	2.25	M112	2.47
M28	5.35	M61	2.66	M113	2.17
M29	3.18	M62	2.97	M114	3.88
M30	2.91	M63	1.84	M115	4.42
M31	2.27	M64	2.63	M117	3.87
M32	2.12	M65	1.94	M118	1.82
M33	2.59	M66	2.72	M119	2.34

MUTANT NUMBER	IA YIELD g/L	MUTANT NUMBER	IA YIELD g/L	MUTANT NUMBER	IA YIELD g/L	MUTANT NUMBER	IA YIELD g/L
M120	9.96	M153	3.31	M187	0.95	M220	3.71
M121	2.57	M154	2.81	M188	2.49	M221	0.13
M122	2.66	M155	5.14	M189	3.03	M222	0.05
M123	3.3	M156	3.29	M190	4	M223	0.01
M124	3.83	M157	2.97	M191	3.37	M224	0.28
M125	2.12	M158	1.46	M192	1.84	M225	0.04
M126	3.72	M159	2.58	M193	0.55	M226	0.31
M127	6.93	M160	0.85	M194	0.51	M227	0.58
M128	3.66	M161	9.97	M195	2.57	M228	0.07
M129	2.54	M162	8.5	M196	3.7	M229	0.01
M130	2.61	M163	3.09	M197	2.85	M230	1.86
M131	2.28	M164	3.44	M198	3.53	M232	0.05
M132	2.22	M165	3.19	M199	3.51	M233	0.61
M133	2.74	M166	3.16	M200	3.75	M234	0.61
M134	3.3	M167	3.37	M201	2.14	M235	0.21
M135	2.62	M169	4.57	M202	3.21	M236	0.43
M136	2.55	M170	4.13	M203	1.55	M237	3.79
M137	4.51	M171	4.26	M204	1.48	M238	0.92
M138	3.94	M172	0.67	M205	3.71	M239	2.36
M139	2.74	M173	4.37	M206	3.28	M240	1.18
M140	3.89	M174	3.92	M207	5.9	M241	0.11
M141	3.67	M175	2.59	M208	2.41	M242	0.01
M142	3.35	M176	4.26	M209	4.82	M243	0.51
M143	4.51	M177	2.69	M210	4.3	M244	0.18
M144	2.94	M178	4.59	M211	6.57	M245	0
M145	3.87	M179	4.14	M212	3.37	M246	0.18
M146	4.01	M180	3.05	M213	2.42	M247	0.64
M147	3.91	M181	3.71	M214	1.98	M248	2.37
M148	2.07	M182	3.29	M215	3.16	M249	0.13
M149	1.83	M183	3.22	M216	0	M250	0.01
M150	4.06	M184	4.27	M217	0		
M151	4	M185	0.14	M218	1.26		
M152	3.51	M186	2.76	M219	0		

\* Mutants highlighted in red are progeny of 49-22 and in blue are progeny of 49-5.

## Appendix 7: Estimation Results for Itaconic acid from RSM

Row	Observed Value	Fitted Value	Lower 95.0% CL for Mean	Upper 95.0% CL for Mean
1	4.00561	4.06852	2.50528	5.63175
2	10.8913	8.40893	6.68048	10.1374
3	4.7717	5.20676	3.39193	7.0216
4	6.5	7.26809	5.53965	8.99653
5	2.03652	4.17586	2.36102	5.9907
6	2.21714	2.95183	1.22339	4.68028
7	0.0	-1.48815	-3.21659	0.240297
8	0.125333	-1.43092	-3.24576	0.383916
9	0.0	0.940574	-0.874264	2.75541
10	3.94166	4.06852	2.50528	5.63175
11	2.00539	0.86425	-0.950588	2.67909
12	1.58161	2.84164	1.11319	4.57008
13	0.256359	1.8781	0.0632588	3.69294
14	11.6875	12.0374	10.2226	13.8522
15	0.0	-1.03404	-2.76248	0.694399
16	0.132082	1.15058	-0.664255	2.96542
17	4.09	4.06852	2.50528	5.63175
18	10.1716	8.40893	6.68048	10.1374
19	6.51048	5.20676	3.39193	7.0216
20	7.4	7.26809	5.53965	8.99653
21	2.31552	4.17586	2.36102	5.9907
22	2.14211	2.95183	1.22339	4.68028
23	0.0	-1.48815	-3.21659	0.240297
24	0.0	-1.43092	-3.24576	0.383916
25	0.0	0.940574	-0.874264	2.75541
26	3.99114	4.06852	2.50528	5.63175
27	1.94656	0.86425	-0.950588	2.67909
28	1.28854	2.84164	1.11319	4.57008
29	0.263891	1.8781	0.0632588	3.69294
30	13.5439	12.0374	10.2226	13.8522
31	0.0	-1.03404	-2.76248	0.694399
32	0.0	1.15058	-0.664255	2.96542

### The StatAdvisor

This **Table** contains information about values of Itaconic acid generated using the fitted model. The **Table** includes:

- (1) the observed value of Itaconic acid
- (2) the predicted value of Itaconic acid using the fitted model
- (3) 95.0% confidence limits for the mean response

## **Appendix 8:** Publications stemming from this study

1. AHMED EL-IMAM, A. & DU, C. 2014. Fermentative itaconic acid production. Journal of Biodiversity, Bioprospecting and Development, 2014.